

The role of adipokines in obesity related beta-cell failure of type-2 diabetes mellitus and endothelial cell dysfunction of cardiovascular diseases

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Abstract

Obesity affects about 520 million people world-wide and more recently studies have shown that fat cells produce proteins called adipokines which have various influences on the human metabolism and has helped to change the perspectives of researchers on the concept of the adipose tissue being just a store of energy. As a result of this, adipokines have been reported to represent a connection between obesity and cardiovascular diseases (CVD) and diabetes mellitus. The concentrations and the bases of the effects of the adipokines in beta cell failure of diabetes mellitus and endothelial cell dysfunction of cardiovascular diseases are still not fully understood.

The effect of leptin and adiponectin, which are two adipokines with opposing effects, has been explored in this study. In the present study, therefore, the concentrations of leptin and adiponectin with significant effect on beta cell and endothelial cell function and the basis of these functions were explored. Also, attempts were made in the present study to correlate the concentrations of leptin and adiponectin with possible clinical pointers to complications.

In order to achieve this, beta cells (BTC) were grown, made into pseudo-islets (which are said to produce more insulin) and treated with various concentrations of leptin and adiponectin and cells assayed for insulin and amylin (to investigate

the role of amylin in insulin secretion). Also the cells were collected and mRNA extracted from these cells, reverse transcription PCR carried out to find out the role of protein phosphatase 1 (PP-1) in the effect of leptin on insulin secretion. PP-1 is a substrate that increases insulin secretion by allowing calcium influx into the cell and is said to be blocked by leptin). Leptin at 500ng/ml was found to significantly ($p<0.05$) inhibit the secretion of insulin and the expression of PP1 gene, thus supporting this as a basis for the effect of leptin on insulin secretion. Adiponectin however increased insulin secretion significantly but was not as consistent in its effect as leptin was in inhibiting insulin secretion.

In order to explore the role of adipokines in cardiovascular diseases, EAHY human endothelial cells were cultured and treated with various concentrations of adiponectin and leptin both individually and in combinations and cells collected and mRNA extracted in order to carry out a reverse transcription PCR for the expression of angiogenic (TIMP2, TIMP3 and MMP2) genes and atherosclerotic (LPA and LPL) genes. Leptin (1nM) was shown to increase the expression of atherosclerotic and angiogenic genes while adiponectin (100nM) inhibited the expression of the atherosclerotic and angiogenic genes. A combination of leptin and adiponectin caused a reduction in the stimulatory effect of leptin on the expression of atherosclerotic and angiogenic genes. This shows that leptin may predispose to CVD while adiponectin reduces the risk of CVD.

The clinical part of this study involved recruiting 150 patients with diabetes after the ethical approval for the clinical study was granted. The data collected from the patients included their age, sex, race, and physical parameters like the body mass index (BMI). Also blood samples were collected to measure the clinical indicators for CVD and renal function such as cholesterol, HDL levels, eGFR, albumin levels and their retinopathy status checked as these are the common complications seen in diabetic patients. The blood samples were also assayed in the laboratory for leptin and adiponectin levels and the leptin, adiponectin and the leptin/adiponectin ratio (LAR) were then correlated with the laboratory determinants of CVD, renal and retinopathy risks. It was found that the LAR and the leptin levels correlates significantly with the BMI, while the leptin levels were significantly correlated with the risk of nephropathy in diabetic patients while adiponectin levels correlated significantly with a reduced risk for developing CVD. The role of the enzymes in the leptin and adiponectin signaling pathway was also explored and it was discovered that ERK, P38 and AMPK all had roles in the effect of leptin and adiponectin on the expression of atherosclerotic and angiogenic genes.

These data indicate that leptin and adiponectin play significant roles in the beta cell and endothelial cell function and are links between obesity and CVD and diabetes mellitus.

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Abbreviations

Abbreviations

APPL1	DCC-interacting protein 13- α
ATP	Adenosine 5'-triphosphate
BAT	Brown adipose tissue
BMI	Body mass index
cAMP	Cyclic adenosine monophosphate
cAMPK	Cyclic adenosine monophosphate kinase
cDNA	Complementary DNA
CO ₂	Carbon dioxide
CVD	Cardiovascular diseases
DNA	Deoxyribonucleic acid
dNTP	Deoxyribonucleotide triphosphate
EAHY	Endothelial Hybridoma
EDTA	Ethylenediaminetetra acetic acid
eGFR	Estimated glomerular filtration rate.
ERK1/2	Extracellular signal related protein kinase
ELISA	Enzyme-linked immunosorbent assay
FBS	Foetal bovine serum
FFA	Free fatty acid

GAPDH	Glyceraldehyde-3-phosphate dehydrogenase
GLUT4	Glucose transporter 4
HbA1c	Glycated haemoglobin
HBSS	Hanks Balanced salt solution
HDL	High density lipoprotein
HMG co A reductase	3-hydroxy-3-methyl-glutaryl-CoA reductase
HMW	High molecular weight
HRP	Horseradish peroxidase
IDDM	Insulin dependent diabetes mellitus
IFG	Impaired fasting glucose
IGF-1	Insulin-like growth factor 1
IGT	Impaired glucose tolerance
IL	Interleukin
IRS-1	Insulin receptor substrate 1
IRS-2	Insulin receptor substrate 2
K _{ATP}	ATP-activated K ⁺ (KATP) channels
LAR	Leptin-to-adiponectin ratio
LDL	Low density lipoprotein
LMW	Low molecular weight
LPA	Lipoprotein a

LPL	Lipoprotein lipase
JAK/STAT	Janus kinase/signal transducer and activator of transcription
mRNA	Messenger ribonucleic acid
MAPK	Mitogen Activated Protein Kinase
MMP	Matrix metalloproteinases
NF- κ B	Nuclear factor-kappa B
NIDDM	Non-insulin dependent diabetes mellitus
NO	Nitrogen Oxide
OGTT	Oral glucose tolerance test
p38–MAPK	p38 isoform of Mitogen-activated protein kinase
PAI-1	Plasminogen activator inhibitor-1
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PED/PEA e	Phosphoprotein enriched in diabetes/phosphoprotein enriched in astrocyte
PI3-K	Phosphatidylinositol 3-kinase
PIP-3 K	Phosphatidylinositol (3,4,5)-trisphosphate Kinase
PKA	Protein kinase A
PKB(Akt)	Protein Kinase B
PKC	Protein kinase C
PP-1	Protein phosphatase 1

RNA	Ribonucleic acid
ROS	Reactive oxygen species
tPA	Tissue plasminogen activator
T1DM	Type 1 diabetes mellitus
T2DM	Type 2 diabetes mellitus
TIMP	Tissue inhibitor of metalloproteinases
TNF- α	Tumor necrosis factor-alpha
WAT	White adipose tissue

Chapter 1

Introduction

1.0 Background and link of obesity to diabetes mellitus and cardiovascular diseases

Recent statistics show that about 520 million people worldwide are obese (WHO 2014 review) and this statistics is significant in relation to recent research which demonstrates a link between obesity, cardiovascular diseases and type 2 diabetes mellitus (Bravo *et al.*, 2006, Barnes 2011). Further research has shown the adipose tissue to be an active endocrine organ made up of fat cells (adipocytes) which releases proteins called adipokines (Belkina and Denis 2010 and Strienstra *et al.*, 2007). As a result of the reported high concentrations of most circulating adipokines in subjects with large amounts of adipose tissue mass (obesity), it has been suggested that the high concentrations of these adipokines seen could be linked to beta cell failure (Rabe *et al.*, 2008). Also, due the pro-inflammatory effect of some of these adipokines, and because adipokines are a product of adipocytes, it has been suggested that they may represent a connection between obesity and cardiovascular diseases. Some of the first adipokines discovered were leptin and adiponectin (Lange and Rathke 2009). Other adipokines that have been reported include visfatin, resistin, apelin, vaspin, omentin (Lago *et al.*, 2007).

Adiponectin is a 30kDa peptide (Michalakis and Segar 2010) which is anti-atherogenic as it inhibits inflammatory, angiogenic and artherogenic processes (D'iez and Iglesias 2003) by suppressing the multiplication and movement of

smooth muscle cells induced by platelet derived growth factor in damaged endothelial cells (Lang and Rathke 2009), decreases macrophage conversion into foam cells thereby inhibiting the formation of atherosclerotic plaques within the blood vessels. It also decreases the uptake of oxidized LDL and inhibits foam cell formation (Balistreri *et al.*, 2010). It was suggested that low adiponectin concentration as seen in obesity could be linked to type 2 diabetes because low levels of adiponectin leads to an increase in blood glucose due to the fact that there is not enough adiponectin to reduce gluconeogenesis in the liver (Miller *et al.*, 2011). Other mechanisms linking low adiponectin in obesity to diabetes was associated with the fact that the low levels of adiponectin leads to a decrease in insulin secretion (Okamoto *et al.*, 2008), and this leads to lower levels of insulin in the blood. When this was viewed in the light of the fact that adiponectin increases insulin sensitivity (Rabe *et al.*, 2008), together with the fact that reduced concentrations of adiponectin in obesity will further reduce tissue sensitivity to insulin, a reduction in the levels adiponectin in an individual can thus lead to a situation where there is not enough insulin to transport glucose and also not enough sensitivity to insulin, which is what is seen in Type 2 Diabetes. Adiponectin is reported to inhibit insulin secretion at low glucose concentrations and increase insulin secretion in high glucose (Winzella *et al.*, 2004)

Leptin is one of the first adipokines to be discovered. It is 16kDa in size and its pro-inflammatory features results in vascular smooth muscle cell movement, increased inflammatory cytokine production, increased platelet collection and

attachment and atheromatous plaque formation (Beltowski 2006, Stapleton *et al.*, 2008). In relation to type 2 diabetes, leptin inhibits insulin secretion thereby leading to an increase in the blood glucose (Zhao *et al.*, 1998, Ahrens and Havel 1999). It is also reported to protect beta cell *in-vivo* in high glucose concentrations and reduces cell death in beta cells at physiological concentrations (Balistreri *et al.*, 2010).

Most of the previous studies have looked at the effect of either leptin or adiponectin individually (and not in combinations) (Brown *et al.*, 2002, Brown and Dunmore 2007, Madani *et al.*, 2006) on endothelial cells and beta cells and also their effect on monolayer cells and not pseudo islets (for beta cells) and the concentrations at which these adipokines had their effects vary considerably between reports.

Also in the present study, the effect of various concentrations of adipokines alone and in combination on pseudo-islets (pseudo-islets have been suggested by Persaud *et al.* in 2010 to have a better dose-related insulin secretion to glucose in comparison to mono layer beta cells) was studied in order to elucidate the effect the adipokines have individually and in combination at a range of adipokine concentrations which are related to those found in physiological (lean) and pathological (obese) conditions. Also, the study looks at the role of adipokines on amylin in insulin secretion and the insulin: amylin ratio calculated in order to have an idea of how much the adipokine treatment

affects amylin secretion and if there is any impact of adipokines on the insulin: amylin balance as suggested by Ionescu-Tirgoviste *et al.* (2010). Attempts will also be made to correlate the levels of leptin-to-adiponectin ratio (LAR) to complications in diabetic patients clinically.

1.1 Obesity

Obesity is defined as a body mass index (BMI) greater than 30 kg/m². The BMI is calculated as weight in Kg divided by height in meter squared [(Wt (Kg)/ (Ht in meters)²]. BMI correlates strongly with adiposity. The BMI classification by the National Heart, Lung, and Blood Institute for adults (age 18-85) defines an individual as being overweight for BMI between 25 and 29.9, and obese for BMI greater than 30 (Karam and McFarlane 2010). Obesity is defined a condition in which the body mass index (BMI) is equal to or greater than 30 or an individual's waist circumference is greater than 94 cm for men and 80 cm for women (Wang and Nakayama 2010).

1.1.1 Epidemiology of obesity

In 2005, data from the World Health Organization estimated that over 400 million adults all over the world are obese. It was also suggested that the

incidence continues to increase at an alarming rate (Vachharajani and Granger 2009). In the report by the World Health Organization (WHO) in 2008, it was estimated that more than 1 billion adults worldwide were overweight, over 300 million of whom are clinically obese (1.4 billion overweight and over 500 million obese in the 2008 statistics by WHO).

Data from many of the previous studies suggest that there has been a progressive rise in the prevalence of obesity worldwide over the last few decades. In the United States of America in 2000, there was an estimated rise of 61% in obesity (assessed by BMI) in 10 years, while it has been estimated that more than half of the American adults in data collected in the year 2000 were overweight. In 2013, data collected shows that the prevalence amongst adults 20-74 years had tripled since 1962 in the men while it had doubled in the female population (10% -34.4% in men and 15.7%- 36.7 in women) (AHA 2013). This data also showed about 154 million overweight with 78 million of these being obese. The phenomenon was also disturbingly recorded to be on the increase in the younger age group leading to childhood obesity with a 5 fold increase in boys and 3 fold increase in girls since 1971 (4%- 18% in boys and 6.1.-18.2% in girls) (AHA 2013).

Data has shown that obesity is commonest in the western population. It has been shown, however, that the populations with the highest numbers are seen

in United States of America, the Middle East, Europe and the lowest in sub-Saharan Africa and East Asia (Roth *et al.*, 2004).

In the United Kingdom however, data from the Poverty Site (2009), a site dedicated to gathering statistics on social welfare all over the UK, show that the increase in prevalence has not been quite as dramatic and an increase from 14%- 25% in men and 17-26% in women in the period between 1993 to 2012 (in individuals over 16 years) was recorded by public health England in 2013 with Scotland having the highest percentages followed by England, Northern Ireland, Wales and the Republic of Ireland. It is also interesting to note that about 7 million working age individuals (compared to 4 million in 2004) were found to be obese in 2009 (Poverty site 2009). This data show that about 20% of the population in the United Kingdom (UK) is obese with over 25% of the population in Birmingham city being obese. The report further states that the average percentage of the male population in the 15-75 year age range that was overweight is 40% with over 20% being obese with women having a lower statistic for overweight and higher numbers of obese individuals (University of Birmingham 2014). In children in Birmingham and West Midlands, in 2006, there was an average of 12% of the children of primary school entry age (5 years) who were overweight with 10% of these obese, and by the time they left primary school at about 11 years, 40% of these children were either overweight or obese. This is suggested to be a disturbing pattern as childhood obesity tends to last into adulthood, thus leading to an increase in the population of

obese adults and a rise in the number of diabetics and people ending up CVD in the United Kingdom (University of Birmingham 2014).

1.1.2 Structure and function of Adipose Tissue

The adipose tissue is a tissue made up of adipocytes and is a vital source of energy in the postprandial fasting state of mammals. Originally, researchers viewed adipose tissue as a passive storage tissue, but more recently, it has become more apparent that adipose tissue acts like an endocrine gland as it secretes hormones, peptides and cytokines which influences many processes in the human body (Cushman and Rizack 1970 and Yu and Ginsberg 2005)

The two types of adipose tissues are White Adipose tissue (WAT), and Brown Adipose Tissue (BAT). The WAT makes up most of the components of the adipose tissues in the body and is the main source of Free Fatty Acids (FFA) which is used in times of starvation to generate adenosine triphosphate (ATP) via oxidative phosphorylation (Strienka 2007).

The WAT is found in various parts of the body and the largest collection is within the abdomen around the intestines, omentum and around the kidneys, and in the subcutaneous tissue in areas such as the buttocks, thighs and

abdominal wall in the body. Therefore researchers have grouped WAT into the areas where they are found and as such, we have the visceral, muscle, epicardial, perivascular and kidney which are all distinct depots of adipose tissues which have different physiological properties and functions (Balistreri *et al.*, 2010).

Recent studies have shown that the main functions of WAT are the control of metabolism by regulating the balance in energy generation and use, the growth and regulation of adipocyte division and function and sensitivity of tissues to insulin. WAT has also been shown to regulate inflammation because they secrete factors that may predispose to or reduce the risk for inflammation (Balistreri *et al.*, 2010). The amount of WAT present can therefore affect the level of inflammation as there may be pathologies associated with the excessive accumulation of WAT in any part of the body (Trayhurn and Wood 2004). When there is excess WAT in the upper body (central and visceral) as seen in men, there is an increase in inflammatory related cardiovascular conditions, while excess accumulation of WAT in the lower part of the body (peripheral or subcutaneous) as seen in women may result in a rise in metabolic disorders (Trayhurn and Wood 2004).

It has been reported by studies that there is a link between central obesity and inflammation. One of the reasons why central obesity is associated with inflammation has been hypothesized to be as a result of the location of the

adipocytes which is said to be close to the portal circulation which can easily take the FFA mediators of inflammation into the liver, thus affecting metabolism (Balistreri *et al.*, 2010). It was also suggested that the inflammatory markers may not be directly from the adipose tissues, but from the liver. It was also suggested that the adipocytes may produce factors that stimulate the production of inflammatory markers from other organs such as the liver (especially C-reactive protein) (Trayhurn and Wood 2004). Balistreri *et al.*, (2010) however also suggested a more localized cause by suggesting that it is due to the differences in the cell biology and properties of the adipocyte cells in the central region, evidenced by different mediator profiles and expression of several genes observed between the visceral and peripheral WAT and also the difference in various body depots of WAT, hence there is a tendency for the WAT in central obesity to be associated with major risk for pathologies.

Previous studies have shown that brown adipose tissue (BAT) is made up of fewer quantities of fat cells but these have more blood vessels supplying them with more mitochondrial chromagens which is why the colour is brown (Balistreri *et al.*, 2010). It has been suggested that BAT is responsible for non-shivering thermogenesis and this is as a result of a more rapid response to the nervous system (sympathetic) which rather than producing ATP, produces heat. The heat production is from an oxidative phosphorylation which is uncoupled carried out by an uncoupling protein-1 (UCP-1) which is found on the internal mitochondrial membrane and causes a proton leak which leads to an exhaustion of the electrochemical gradient needed for oxidative

phosphorylation. BAT is difficult to find postnatally but it has been located by Positron Emission Tomography (PET) scan to be located in the cervical, supra-clavicular, axillary and para-ventral regions of the body (Virtanen *et al.*, 2009).

1.1.3 Adipose tissue and energy homeostasis

Studies have shown that the adipose tissue is involved in the maintenance of energy levels in such a way that the amount of adipose tissues remains the same without excessive weight loss or gain (Galic *et al.*, 2010). This process is achieved by the regulation of appetite and intake of and weight gain, the use of energy and weight loss. This process is regulated by the hypothalamus via neuropeptide (Klaus 2001).

The process of homeostasis is controlled by the nervous system which consists of contributions from central and autonomic nervous systems. When there is urgent need for energy in states where there are low levels of glucose (starvation), the sympathetic nervous system (SNS) stimulates WAT lipolysis leading to a release of FFA which are metabolized to produce ATP while on the other hand when there is less need for energy, the parasympathetic nervous system (PNS) stimulates the storage of fat as energy (Romijn and Fliers 2005). Balistreri *et al.*, (2010) suggested that the evidence of the effect of the SNS and PNS was seen in the fact that both WAT and BAT have SNS innervations and

β 3 adrenergic receptors. The BAT is responsible for cold-induced shivering thermogenesis and this process involves SNS activity which generates heat from uncoupled oxidative phosphorylation. WAT stimulated by upregulated SNS activity in response to cold is commonly seen in obesity and increases thermogenesis from oxidative phosphorylation of FFA seen in the liver, muscle and fat cells (Balistreri *et al.*, 2010)

Summarizing, research has shown that obesity occurs as a result of conditions in which there is an increase in caloric intake and reduction in calorie use and BAT mass and function are decreased in obese individuals

1.1.4 Pathophysiology of obesity

Obesity is associated with over-nutrition and lack of physical activity. Research has shown, however that there are other issues that are important contributory factors to the pathogenesis of obesity which are linked to diabetes and cardiovascular diseases (Balisteri *et al.*, 2010)

Stress, as one of the factors predisposing to visceral obesity is also said to be caused by obesity (therefore forming a vicious cycle) due to the impairment of metabolic homeostasis of the human body. When there is an increase in White

Adipose Tissue (WAT) in sites which are metabolically active such as the liver and immune cells, there is a release of pro-inflammatory cytokines which influences inflammation. The cytokines released in situations like this are called adipokines (Rabe *et al.*, 2008).

Obesity and stress have been described as relating in a vicious cycle because when obesity induces stress for a prolonged time, the hypothalamic-pituitary-adrenal axis and the central and peripheral components of the autonomic nervous system are stimulated to counter the stress by releasing glucocorticoids (Balisteri *et al.*, 2010). Glucocorticoids are steroid hormones that induce the differentiation of pre-adipocytes into adipocytes that further encourages the growth of mass of WAT which further leads to an increase in the stress stimulus and a further increase in the WAT mass in addition to a chronic state in which there is a high level of inflammatory adipokines release, this being responsible for the link between obesity and cardiovascular disease and diabetes (Balisteri *et al.*, 2010).

Recent studies have shown that in normal circumstances in the WAT tissue, inflammation is mediated as a result of the infiltration by the M1 and the M2 macrophages. The M1 macrophages are suggested to encourage inflammation due to the presence of a surface marker (CD11c+) which causes the release of iNOS and other pro-inflammatory cytokines while the M2 macrophages have been shown to reduce inflammation by inhibiting cytokines that encourage

inflammation such as interleukins by the release of arginase (Striensa 2006). In the normal state, WAT is in an environment that is anti-inflammatory as a result of the actions of the M2 macrophages and also due to the activities of the peroxisome proliferator-activated receptor (PPAR- α and - γ) and liver X receptor (LXR) which are all involved in the transport of transport and metabolism and able to antagonize inflammatory activity (Balistreri *et al.*, 2010).

It is therefore important to consider why there is a link between WAT and inflammatory conditions if the WAT environment is designed to be anti-inflammatory. It has therefore been suggested that it is as a result of the WAT cellular composition restructuring which involves an increase in the number of cells (hyperplasia) and size (hypertrophy) of adipocytes and macrophage infiltration and the resulting fibrosis within the WAT tissue in inflammatory states related to obesity (Treyhurn and Wood 2004).

Adipocyte hypertrophy is one of the important factors that tilt the WAT environment into a pro-inflammatory one. The hypertrophy occurs when there is an increase in deposition of fat in adipocytes that are fully differentiated, and when there is a rise in the expression of inflammatory mediators. Hypertrophy in adipocytes tends to influence the immune system to produce more pro-inflammatory cytokines (Balistreri *et al.*, 2010) and the change in the cytokine profile leads to the modification of the M2 macrophages around the WAT to M1 macrophages which are more pro-inflammatory. Also because of the

differentiation of the monocyte recruited by the WAT, there is an increase in the number of M1 macrophages (Striensa *et al.*, 2006). In a condition where there is high adiposity, studies have shown that there is an increase in the infiltration of the inflamed visceral WAT by macrophages which leads to production of cytokines which bind to receptors and aid the production of adhesion molecules that affect endothelial cells by attracting more macrophages and monocytes to the endothelial cells as a result of the inflammatory WAT cytokine profile (M1 to M2). This also forms the basis for the relationship between angiogenesis and adipogenesis (Sell and Eckel 2010).

1.1.5 Adipokines and role of adipokines in type 2 diabetes and cardiovascular diseases

Adipokines are protein molecules released by the adipocytes and are molecules that are biologically active and that have been shown to mediate various stages of metabolism. Adipokines help to mediate the regulation of food consumption, the metabolism of glucose, protein and lipids, blood pressure regulation, modulation of inflammation and immunity, and they work together to regulate inflammation, insulin action, and glucose metabolism locally and systemically (Striensa 2006). In an obese person, the adipokine/cytokine network is altered leading to a state that encourages inflammation and the impairment of adipocyte metabolism (Treyhurn and Wood 2010).

Adipokines that stimulate inflammation such as pro-inflammatory cytokines, chemokines, molecules associated with thrombosis, and hypertension are produced in several pathways and these are initiated by stressors from both within and outside the cells. The leading stressor from within the cell are the free fatty acids (FFA) which are chronically increased in obesity as a result of insulin becoming limited in its ability to inhibit lipolysis and consumption of dietary lipids in excess (Trayhurn and Wood 2004). It has been suggested by previous studies that there are receptors in WAT called toll like receptors (TLR-4) and their expression is increased in obese individuals and these receptors mediate inflammatory processes (Balistreri *et al.*, 2010). Factors that activate TLR-4 include FFA, hypoxic conditions in obesity, and lipopolysaccharides (LPS). LPS is found in high levels in subjects consuming a high-fat diet, and is continually being produced when the gut gram-negative bacteria die from where it is taken up into the intestinal capillaries and they move within lipoproteins in the blood around the body. Thus gut bacteria have been linked to chronic obesity-related inflammatory conditions (Waki and Tontonoz 2007).

The activities of FFA in mediating inflammatory conditions via TLR-4 are thought to involve the activation of macrophages (especially the CD11c+ subset) via the TLR-4 pathway. The inflammatory state in WAT also involves a paracrine loop between adipocytes that are hypertrophied and macrophages (Balistreri *et al.*, 2010). This loop makes macrophages secrete pro-inflammatory TNF- α and this acts via the TNF- α receptor 1 subtype. This mediates a process

of inflammation in adipocytes that are hypertrophied and increase the release of FFA.

The FFA generated is metabolically active and may feedback to influence the adipocytes leading to an inflammatory response and adipokine overproduction via the TLR pathway (Vacharajani and Granger 2009). Within the adipocytes, the mitochondria and the endoplasmic reticulum appear to be sensitive to metabolic stressors (Rockl *et al.*, 2008). Furthermore, hypoxia has been reported to be a strong inflammatory factor in WAT in obese states. This is as a result of an increase in reactive oxygen species which cause oxidative stress as mentioned earlier in the present study. All the factors related to inflammation culminate predominantly in the activation, principally via TLR4 receptor, of NF- κ B (nuclear Factor kappa-light-chain-enhancer of activated B cells) transcriptional factor, responsible for the production of inflammatory mediators (Sell and Eckel 2010).

With regards to the process involved in the development of obesity, it has been reported by previous studies that there is an interaction between genes and the environment (diet and physical activity). Genetically there has been a description of a rare, monogenic disorder associated with obesity with a suggestion that the inheritance is polygenic. Due to the fact that more recently, food has become more affordable and is now more available, and also the fact

that more people are becoming sedentary, the incidence of obesity is on the increase (Draznin and Marakhovski 1973)

Some environmental factors may have genetic components such as the variations in individual metabolic rate which has been explored emphasizing the thermic effect of food, and non-exercise activity thermogenesis as being important in the caloric expenditure (Rockl *et al.*, 2008). Previous studies have not been able to come to an agreement as to whether the increase in obesity seen is mainly due to an increase in food intake or a decline in physical activities or an equal contribution from the 2 factors. Early childhood nutrition, sleep deprivation, infection and stress are all addition environmental factors that may contribute to obesity (Roth *et al.*, 2004).

The theory behind many of the studies suggest that evolution is in favour of storage of energy and therefore in an environment where there is plentiful, there is likely going to be an increase in energy storage in the form of obesity (Schwatz and Porte 2005). Previous studies have suggested that in the brain, mechanisms include changes in behavior (in appetite, food intake, and physical activity), changes in autonomic function, leading to effects on thermogenesis and on metabolism. Also it has been suggested that some of the other factors that have impact on development of obesity are seen in changes in the thyroid and neuroendocrine functions (Schwartz and Porte 2005, Draznin and Marakhovski 1973).

Centres in the brain such as the hypothalamic satiety centre and the feeding centers get feedback from the periphery in conditions such as starvation (feedback is via signals like, glucose, and fatty and amino acids) satiety signals (the satiety signals are cholecystokinin, peptide-YY, ghrelin, leptin, glucagon-like peptide-1, and amylin, as well as vagally mediated neural signals). Also there is another pathway called the hedonic pathway with signals originating from the olfactory, taste, visual, and tactile senses (Lutter and Nestler 2009). Lutter and Nestler (2009) also noted the differences between circuits involved in homeostatic (appetite) and hedonic pathways involved in addiction, with neurons involved in metabolic pathways as well as those related to hedonic centers expressing leptin receptors, suggesting that both are important in understanding obesity.

Leptin is a fat derived adipokine and it was thought to originally function as an endogenous anti-obesity factor as it is one of the response stimulus sent to the brain in times of abundance to suppress food intake and stimulate metabolic processes to use up the excessive energy stores (Lutter and Nestler 2009). It has been reported by more studies that there can be resistance to the actions of leptin seen in obesity. The consequence of this is that the inhibitory effect of leptin in food intake is reduced and the body develops a state of “insatiety” (Gertler 2009).

The concept of leptin resistance in obese individuals has been shown to be due probably to the circulating factors that antagonize its actions, or a reduction in the blood-brain barrier transport of leptin (Flier *et al.*, 1987).

1.1.5.1 Leptin and its role in cardiovascular diseases and beta cell failure

1.1.5.1.1 Structure and function of Leptin

Leptin is one of the protein molecules released by the adipocytes specifically from the white adipose tissue (WAT) which are biologically active and have been shown by previous studies to mediate metabolism in the body (Dardeno *et al.*, 2010). Structurally, Leptin is made up of 167 amino-acids and has a four bundle motif (4 alpha helices) and 2 short beta strands, which is similar to the structure of cytokines and has a molecular weight of about 16-kDa and is encoded by the OB (*lep*) gene (Dubey and Hesong 2006).

In the human body, leptin secretion has a circadian rhythm which is pulsatile with a pattern of producing the highest leptin levels in the period between midnight and early part of the morning and lowest levels are seen in the early mornings to the middle of the afternoon. Also studies have shown that leptin levels may be as high as 75.6% higher than levels seen in the afternoon during

the night. The rhythms of leptin secretion in obese and lean individuals are similar except in some obese individuals that have been shown to have higher pulse amplitudes (Dardeno *et al.*, 2010).

The *in-vivo* function of leptin includes the regulation of food taken in and the body weight, the reduction of appetite, and the regulation of energy use by increasing the sympathetic activation leading to an increase in the use up of energy and a subsequent reduction in body weight and the size of adipose tissues (Bravo *et al.*, 2006). Due to the fact that leptin is increased after long periods of overfeeding, the homeostatic control mechanism of leptin suggest that leptin in *in-vivo*, is an anti-obesity hormone but research has shown that this effect of leptin doesn't exist in most obese individuals due to a state of leptin resistance that leads to high levels of leptin in obese individuals (Yu *et al.*, 2004).

The role of leptin in the development of CVD involves the activation of the sympathetic nerve system activation, the regulation of renal blood vessel control, the general blood vessel tone, and the control of blood pressure (Bravo *et al.*, 2006). Leptin has been shown to promote the oxidation of fatty acid in the liver, pancreas and skeletal muscle and also modulates hepatic gluconeogenesis and pancreatic beta cell function (Rabe *et al.*, 2008)

1.1.5.1.2 Leptin Signalling Pathway

Leptin has its effects by binding to a receptor called the leptin receptor (ObR) which is encoded by the db gene and has been shown by studies to be a member of the cytokine receptor superfamily called the class I (Dubey and Hesong 2006). The ObR gene is expressed as short (a,c,d,f with 30-40 cytoplasmic residues) and long isoform (ObRb with 300 cytoplasmic residues) (Fruhbeck 2006). The ObRB is the functional form of ObR (ObRb) and is expressed mostly in the hypothalamus, and in endothelial and the immune system cells (Dubey and Hesong 2006). ObRb is considered to be of major importance for leptin signalling, with full signalling capacity being achieved when leptin activates the mitogen-activated protein (MAP) kinase and Janus kinase/signal transducer and activator of transcription signalling pathways (JAK/STAT pathway) (Dubey and Hesong 2006). ObR a and c are expressed in the vessels surrounding the brain and are said to be responsible for the uptake of leptin into the brain during signalling from the body. ObRe encodes a soluble receptor and has been reported to act as a buffering system for free circulating leptin (Fruhbeck 2006).

After leptin binds to ObRb, there is activation of JAK2 (Janus kinase 2) by autophosphorylation and this leads to activation of Ob-Rb by phosphorylation at Tyr⁹⁸⁵ and Tyr¹¹³⁸. Phosphorylation at Tyr¹¹³⁸ further leads to the binding to STAT3 (Signal Transducer and Activator of Transcription 3) which is further phosphorylated and activated by JAK2 to form activated STAT3. Activated

STAT3 then forms homodimers which is able to move into the nucleus to influence gene transcription of the target angiogenic genes such as TIMPs and MMPs (especially TIMP1) (Handy *et al.*, 2011). As seen in figure 1.1 leptin influences the regulation of NF- κ B induced genes and with high leptin levels as seen in obesity this may lead to incorrect regulation and a state of inflammation. Figure 1.1 also shows that leptin increases gene expression for VEGF and influences the IRS pathway thereby leading to cell migration and invasion which is one of the components of the process of atheromatous plaque development in the endothelium. A combination of all these effects of leptin described above provides evidence for the role of leptin in the predisposition to cardiovascular diseases. Figure 1.1 also shows that leptin reduces Ppp1ca gene expression which codes for the PP1 thereby reducing calcium influx into beta cells and the release of insulin. Also leptin, via the IRS pathway influences GLUT 4 translocation and the uptake of glucose in target tissues. These effects of leptin suggest a link between adipokines (in obesity) and type 2 diabetes mellitus.

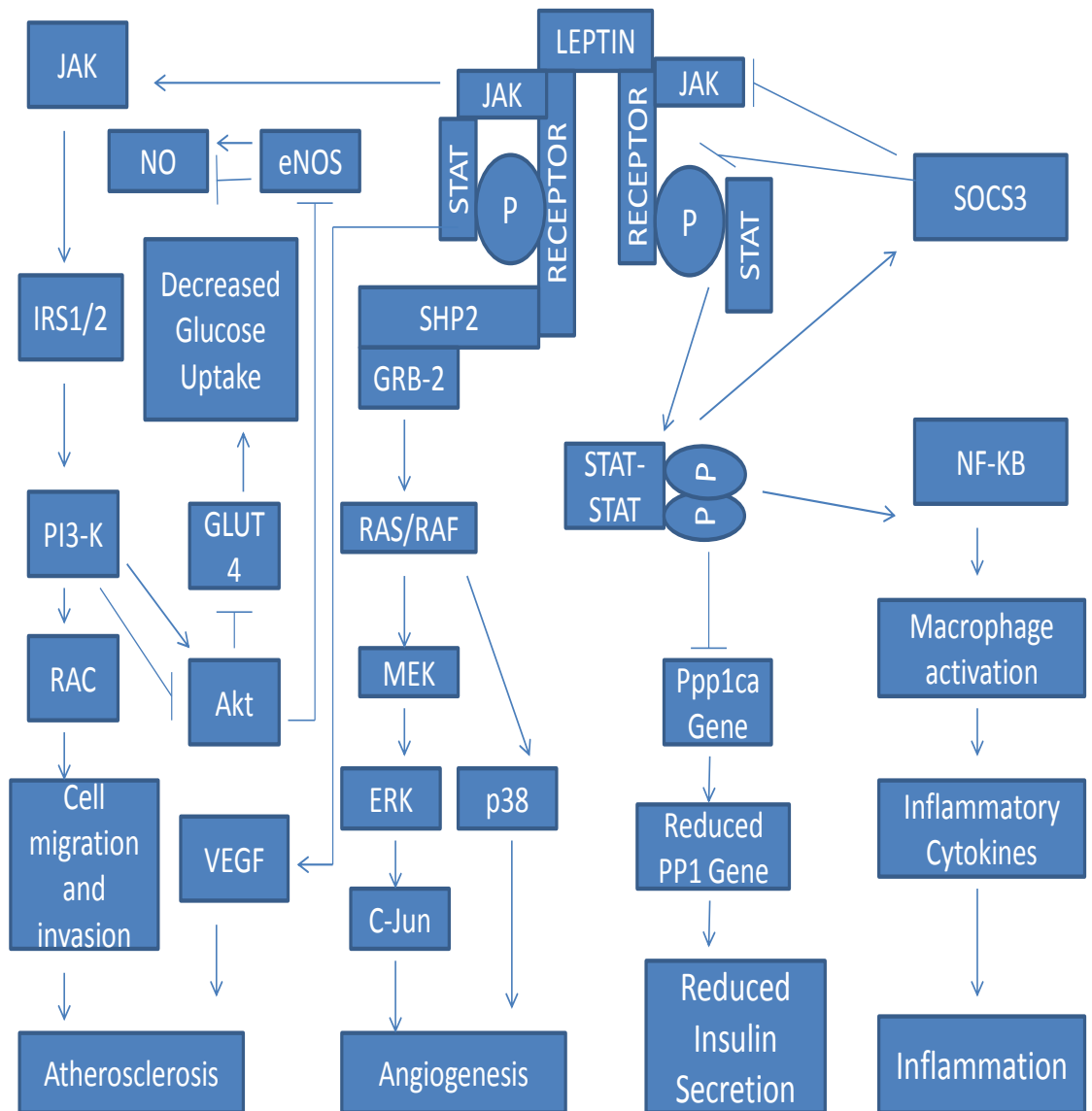


Figure 1.1 Leptin Signalling Pathway. Leptin Influences insulin secretion via IRS and endothelial cells via its influence on ERK and STAT. It also influences gene expression in CVD via the JAK-STAT pathways. Modified from Kanehisa Laboratories 2013)

1.1.5.1.3 Role of leptin in CVD

Studies have suggested that most of the pathogenic role of leptin in CVD involves the formation of atheromatous plaque and thrombus in the cardiovascular system (Dubey and Hesong 2006). Leptin has been linked to the development of CVD via a process that involves a trigger factor (trauma to endothelium or myocardium by friction, elevation of blood pressure, high levels of cytokines) which leads to compensatory mechanisms such as inflammation (which can be exaggerated), angiogenesis in an attempt to repair the damage, the deposition of smooth muscle cells and fat cells (in dyslipidemia) which forms the basis of atherosclerosis (Wang and Nakayama 2010, Dubey and Hesong 2006).

In terms of inflammation, it is suggested that leptin plays a controversial role in endothelial cell function as studies demonstrate that leptin at high concentrations elicits endothelium-dependent NO-mediated vasorelaxation in non-obese rats (Sweeney 2010). However, it has been suggested that leptin may increase NO synthase which increases the amounts of NO production, thereby leading to the impairment of endothelial function and the promotion of atherogenesis by inducing oxidative stress (Koh *et al.*, 2008). When it is exposed to free radicals generated from oxidative stress, NO may undergo conversion to toxic molecules such as peroxynitrite that impair endothelial function. Furthermore, research has also shown that leptin at concentrations

found in obese people (“obese-range” concentrations), (but not at low physiological concentrations) impairs NO-dependent vasorelaxation induced by acetylcholine both *in vitro* and *in vivo*, an effect that has not been demonstrable in non obese individuals (Koh *et al.*, 2008). In order to understand this better, further research has been done and this suggests that leptin promotes the secretion of tumour necrosis factor and interleukin 2 and 6 and also facilitates the accumulation and production of reactive oxygen species (ROS), and the enhancement of the production of monocyte chemo-attractant protein-1 which further stimulates production of pro-inflammatory cytokines (Dubey and Hesong 2006).

In the process of the repair of an injury to the endothelium, leptin stimulates migration and multiplication of vascular smooth muscle cells and the expression of matrix metalloproteinase-2 (Park *et al.*, 2001). In addition to this, leptin has been shown to enhance the secretion of endothelin-1 and increase sympathetic nervous activities in the kidneys and adrenals of rats that cause high blood pressure as a result of hyperleptinemia in obese rats (Dubey and Hesong 2006). In addition to calcification of blood vessels, in conditions such as diabetes where there is beta cell dysfunction and hyperglycemia, leptin stimulates lipoprotein lipase (LPL) secretion in macrophages and in high glucose conditions, increases the collection of cholesterol esters in foam cells in the body (Koh *et al.*, 2008). However, other studies have suggested that at normal glucose levels, leptin may prevent the accumulation of cholesterol within macrophages. Leptin has also been shown to promote the clearance of hepatic

HDL by increasing the scavenger receptor type B1, thereby decreasing plasma HDL level. This suggests that when there is high levels of glucose, leptin may inhibit cholesterol removal from peripheral tissues by reducing the concentration of HDL thus affecting the local cholesterol balance in diabetic patients (Koh *et al.*, 2008)

With regards to the heart, when there is damage to the endocardium, there may be formation of a thrombus which when infected can form a vegetation in the heart and embolism of this can lead to stroke, pulmonary hypertension and other complications such as ischaemic heart disease from embolism. Leptin is suggested to contribute to a reduction in oxidation rates of fatty acid (the first observable abnormalities in failing hearts) in the hearts of obese individuals who tend to have high levels of leptin (Sweeney 2010). Leptin stimulates oxidation of fatty acids via a pathway that involves STAT3, NO and MAPK (Sweeney 2010). High levels of Leptin may play a part in platelet hyperactivity, hypercoagulability and a shift in the coagulation-fibrinolysis balance to a pathologic pattern, thus predisposing to thrombosis (Koh *et al.*, 2008). Leptin is also suggested to be negatively inotropic and also to stimulate hypertrophy of cardiomyocytes associated with MAPK activation which is p38 dependent (Karmazyn *et al.*, 2009).

1.1.5.1.4 Role of Leptin in beta cell Failure

Studies to explore the role of leptin in the process of developing diabetes reveals that this involves a decrease in insulin secretion from beta cells and also the development of insulin resistance (Seufert 2004).

As seen in figure 1.1, Akt is the kinase that phosphorylates GLUT-4 (Glucose transport protein-4) and helps its translocation to the cell membrane where it binds to the cell membrane and allows the diffusion of glucose from the blood into the cell via tubules on the GLUT-4(Wilcox 2005). Therefore when leptin binds to its receptor, it dysregulates IRS proteins by the inhibition of the phosphorylation of IRS-1, thus leading to a chain reaction that leads to a decrease in the phosphorylation of Akt and subsequently a decrease in the phosphorylation of GLUT-4 which helps to transport glucose into target cells, thus leading to insulin resistance (Laakso 2010).

With regards to insulin secretion, numerous studies suggest that leptin inhibits insulin secretion by a direct action on the beta cells mediated by activation of PI3-kinase, and opening of ATP-regulated K⁺-channels, by impairment of the cyclic AMP-PKA-pathway and perhaps in the longer term, by reduction of beta cell lipid content. Studies have also suggested that leptin suppresses prepro-insulin mRNA in the pancreas thus leading to less insulin secretion (Seufert 2004).

1.1.5.2 Adiponectin and its role in beta cell failure and cardiovascular diseases

1.5.5.2.1 Structure and function of adiponectin

Adiponectin (also called GBP-28, apM1, AdipoQ and Acrp30) is an adipocytokines or adipokines which are produced by adipocytes and help to modulate metabolism (Goldstein and Scalia 2004). Adiponectin is a large protein with a molecular weight of about 30 kDa (Acrp30). Adiponectin is a 244-amino acid protein and its origin is exclusively from adipocytes in white adipose tissue and is shown to be a product of the APM1 (which is the adipose tissues most abundant gene transcript 1), located on the chromosome 3q27, close to the site of the genes responsible for type II diabetes and adiposity (Barseghian *et al.*, 2011). In the human body, adiponectin has been shown to be one of the most abundantly secreted adipokines and corresponds to 0.05% of the proteins in the serum in the human body. Researchers have found serum levels of between 3-30 µg/mL in humans and 3-6 µg/mL in rodents (Michalakis and Segars 2010). In the plasma, adiponectin circulates mainly as a full length adiponectin (fAd) and a smaller globular fragment (gAd) (Adya *et al.*, 2012). Adiponectin is secreted by adipose tissue in here forms; in the form of one trimer which is a low-molecular-weight (LMW) form, a combination of two trimers as a middle-molecular-weight (MMW) type, or as six trimers in a high-

molecular-weight (HMW) form and circulates either as a trimer or an oligomer (Michalakis and Segars 2010).

The formation of high molecular weight (HMW) multimers depends on the disulfide bond between Cys residues at the amino terminus. Studies have also suggested that a truncated globular domain form of adiponectin is generated by proteolytic cleavage. The globular domain of adiponectin (gAd) appears more potent than the full-length protein in at least some actions (Waki and Tontonoz 2007).

As seen for leptin, men have lower adiponectin levels than women. It is also lower in obesity, diabetes and coronary artery disease. The effect of adiponectin are mediated via receptors AdipoR1, AdipoR2 and t-cadherin. AdipoR1 has been shown to be expressed in many tissues unlike the AdipoR2 which is expressed most abundantly in the liver. Studies have shown that AdipoR2 stimulates the release of energy by increasing the oxidation of fatty acid and inhibits oxidative stress and inflammation (Rabe *et al.*, 2008).

AdipoR1 has been shown to have a high affinity for the gAd receptor and very low affinity for fAd, and AdipoR2 has intermediate affinity for both types of adiponectin form. While AdipoR1 is expressed in large amounts in skeletal muscle and at moderate levels in other tissues, AdipoR2 is predominantly

expressed in the liver (Goldstein and Scalia 2004). These findings are consistent with the observation that fAd has a greater effect on hepatic metabolic signaling, whereas both gAd and fAd elicit metabolic effects in skeletal muscle. Aortic endothelial cells express both adiponectin isoforms, but appear to preferentially express mRNA for AdipoR1, suggesting a signalling role for gAd in this cell type (Goldstein and Scalia 2004).

Adiponectin has been shown to inhibit the gluconeogenesis and oxidation of fatty acid in the liver. Studies have also shown that adiponectin stimulates insulin secretion and increase the uptake of glucose by skeletal muscle and to mediate food intake and energy use (Galic *et al.*, 2009).

1.1.5.2.2 Role of adiponectin in cardiovascular diseases and beta cell failure

Adiponectin influences the Pathogenesis of CVD via the following pathways;

Activation of AMP Kinase (AMPK)

Inhibition of oxLDL-Induced Superoxide Production

Endothelial Apoptosis

1.1.5.2.3 Anti-inflammatory effects of adiponectin

It has been suggested by previous studies that when adiponectin binds to adiponectin receptors on the endothelial cells, it stimulates phosphatidylinositol-3 (PI-3) kinase which stimulates PDK-1 (Phosphoinositide dependent kinase 1) activation. PDK-1 is required to activate Akt. Akt is responsible for phosphorylation of eNOS leading to increased production of NO (Nitric Oxide) which is responsible for its anti-inflammatory effect on the endothelial cells (Goldstein and Scalia 2004). Nitric oxide inhibits the increase of endothelial cell adhesion molecules induced by cytokines (E-selectin, VCAM-1) thus reducing the risk of CVD. NO also reduces the risk of CVD by relaxing the blood vessel and thus reducing the chance of an injury from high blood pressure (Vachharajani and Granger 2010). Previous studies have also shown that adiponectin reduces lipid accumulation, the reduction of the expression of scavenger receptors in macrophages, and encourages macrophage polarization, which all results in its anti-inflammatory activities and helps to reduce the risk of CVD (Laakso 2010).

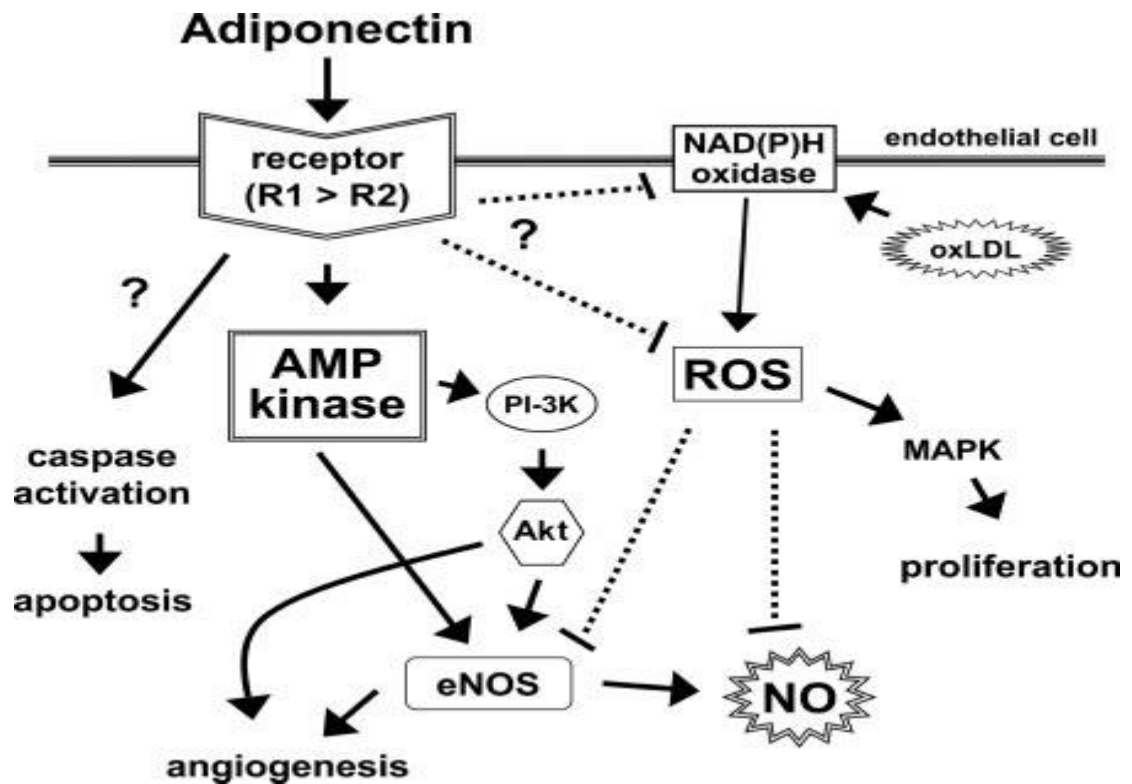


Figure 1.2 Effect of adiponectin on endothelial cells. Adiponectin stimulates NO synthesis and angiogenesis. Adiponectin also encourages cell proliferation via the MAPK pathway. (Modified from Goldstein and Scalia 2004)

1.1.5.2.4 Adiponectin and its effects on Vascular smooth muscle proliferation/angiogenesis

It has been suggested in previous studies that adiponectin may modulate inflammatory signalling in endothelial cells via the influence of interaction between the cAMP-protein kinase A and nuclear factor-B pathways (figure 1.2). As oxLDL-induced super-oxide generation in endothelial cells is linked to an

NADPH (nicotinamide adenine dinucleotide phosphate) oxidase pathway, the suppression of this process by gAd may involve regulation of the activity of certain isoforms of NADPH oxidase or its protein subunits in the vascular cells (Golstein and Scalia 2004). This pathogenesis involves adiponectin binding to the AdipoR receptor leading to the inhibition of the NADPH oxidase and a decrease in the ROS (reactive oxygen species) activity due to a decrease in the production of ROS. A decrease in ROS activity leads to a removal of the inhibitory effect ROS has on NO production from eNOS and also the inhibitory effect on angiogenesis, thus leading to vascular relaxation and angiogenesis. The effect of ROS inhibition leads also to a reduction of MAPK production which leads to a decrease the cellular proliferatory effect of MAPK. All these activities result in angiogenesis and vascular dilation and a reduction in risk of CVD (Goldstein and Scalia 2004).

1.1.5.2.5 Adiponectin and atherosclerosis

Adiponectin has been shown in previous studies to have direct anti-inflammatory and anti-atherosclerotic effects. Adiponectin inhibits TNF- α -induced inhibition of nuclear factor-kappa B (NF- κ B)-alpha (I κ B- α) phosphorylation and sub-sequent NF- κ B activation, as well as the expression of monocyte adhesion molecules in human aortic endothelial cells (Galic *et al.*, 2009). It has also been shown that this effect inhibits the ability of monocytes to adhere to endothelial cells, a key step in the formation of the atherosclerotic

lesion thus leading to a reduction in the risk of CVD. Adiponectin also inhibits the expression of the class A macrophage scavenger receptor and lipid accumulation in human macrophages derived from monocytes, and inhibits smooth muscle cell proliferation (Zhu *et al.*, 2008).

Furthermore, adiponectin is suggested to favour plaque 'stabilization' by increasing IL-10 (interleukin-10) secretion and subsequent production of TIMP-1 (tissue inhibitor of metalloproteinases-1). Also the proliferation of VSMC (vascular smooth muscle cell) induced by growth factors can also contribute to atherogenesis. In cultured VSMCs, adiponectin has been shown to suppress VSMC proliferation and migration via direct binding to PDGF-BB (platelet-derived growth factor-BB) and generally inhibits growth-factor-stimulated ERK (extra-cellular-signal-regulated kinase) signalling in VSMCs (Okamoto *et al.*, 2008).

1.1.5.2.6 Adiponectin and reduction in apoptosis of endothelial cells

It has been suggested from previous studies, that HMW multimers of adiponectin suppresses apoptosis of endothelial cells and this is shown in its effect on caspase-3 activity in human umbilical vein endothelial cells through the AMPK pathway (Waki and Tontonoz 2007)

1.1.5.2.7 Role of adiponectin in Type 2 Diabetes

The role of adiponectin in the development of diabetes has been studied. It has been shown that the structure of adiponectin which is made up of a globular adiponectin which is in the form of a trimer, and the full-length adiponectin which is produced as 3 species of multimers: an LMW trimer, an MMW hexamer, and an HMW multimer. Studies have also shown that of the roles of adiponectin in diabetes is carried out by binding the receptors. The inhibition of AdipoR1 by RNA interference leads to an inhibition of the binding of globular adiponectin while the inhibition of AdipoR2 by RNA interference inhibits full-length adiponectin-specific binding (Adya and Randeva 2012).

The role of adiponectin in diabetes is said to be mediated by AMPK or PPAR and in previous studies where these have been inhibited, it has been shown that there was partial reduction in adiponectin-stimulated glucose uptake. It has also been shown that the overexpression of AdipoR1/R2 in liver cells have led to PPAR or AMPK activation and fatty-acid oxidation. It has also been shown that adiponectin reduces the levels of glucose in the plasma. These studies support the conclusion that AdipoR1 and AdipoR2 serve as receptors for globular and full-length adiponectin and mediate increased AMPK, PPAR ligand activities, p38 MAPK, and biological functions of adiponectin (Kadowaki *et al.*, 2006).

It has been shown that adiponectin levels are decreased in obese humans despite increased fat mass and hypoadiponectinemia has been seen to correlate with insulin resistance in most circumstances (Wilcox 2005)

The role of adiponectin in glucose metabolism is said to be seen mostly seen in the liver and skeletal muscles and adiponectin has been shown to increase AMPK phosphorylation and via this pathway it stimulates glucose uptake and the oxidation of fatty acid in the muscle, and reduces the expression of enzymes involved in gluconeogenesis such as phosphoenolpyruvate carboxykinase (PEPCK) and glucose-6-phosphatase in the liver (Waki and Tontonoz 2007).

In other studies into humans, plasma adiponectin levels were correlated negatively with adiposity, insulin resistance (type 2 diabetes), and metabolic syndrome and positively correlated with markers of insulin sensitivity in frequently sampled intravenous glucose tolerance tests. It was also suggested that in addition to its insulin-sensitizing effects, adiponectin may alter glucose metabolism through stimulation of pancreatic insulin secretion *in vivo* (Rabe *et al.*, 2008).

The role of lowered adiponectin in the pathogenesis of diabetes involves its glucose-lowering effect due partly to its activation of the AMP-activated protein

kinase (AMPK) cascade and also AMPK mediated the catabolism of existing intracellular stored energy reserves, such as triglycerides and glycogen and an insulin-independent influx of energy sources outside the cells as seen in the influx of glucose. Adiponectin has also been shown by research to sensitize tissues to insulin and reduce inflammation. Because of the link between inflammation and obesity and the development of diabetes, adiponectin may reduce the risk of development of both diabetes and atherothrombotic diseases (CVD). Another potential mechanism for adiponectin's protective effect is improved insulin secretion, in that it has been recently shown to counteract cytokine and fatty acid induced cell dysfunction (Duncan *et al.*, 2004).

1.1.5.3 Effects of leptin and adiponectin in combination

Leptin and adiponectin are both important for the maintenance of appropriate insulin sensitivity and overall energy balance. The effect of both leptin and adiponectin has been studied individually but little is known about the potential interactions when they are combined. Research reveals that elevated leptin concentrations reduced adiponectin signalling and metabolic effects in skeletal muscle cells, while hyperleptinaemia may be an important contributory factor leading to adiponectin resistance in skeletal muscle, thus exacerbating the disturbed metabolic milieu. Handy *et al.* (2011) suggested that adiponectin inhibits the function of leptin by inhibiting leptin-stimulated activation of Jak2, and as Jak2 activation represents the most upstream event in leptin signal

transduction, this inhibition was suggested to be the basis for the interaction of leptin and adiponectin in beta cell function. Research has also shown that protein tyrosine phosphatase 1B (PTP1B) dephosphorylates and thus deactivates Jak2, acting upstream of Ob-Rb and Stat3 to negatively regulate leptin signal transduction. PTP1B is an important inhibitor of leptin signalling in the hypothalamus, where PTP1B participates in the control of glucose and fat metabolism (Zhu *et al.*, 2008).

Leptin induces expression of Supressors of Cytokine Signaling 3 (SOCS-3), which in turn negatively regulates leptin signalling. SOCS-3 inhibits leptin signalling by binding Ob-Rb and preventing Jak2 phosphorylation of Stat3, and targeting the activated receptor complex for degradation. Adiponectin inhibits leptin-stimulated formation of TIMP-1/MMP-1 complexes which stimulate fibrinogenesis (Handy *et al.*, 2011).

1.2 Diabetes: Epidemiologic impact of Diabetes

Diabetes affects about 3-5% of the western population, and has been described as one of the major health issues of this century (Laakso 2010). In 2001, the global estimate of prevalence of people with diabetes was 151 million and this number is estimated to have increased to 221 million by 2010 (representing a 46% increase) (Laakso 2010). More recent statistics put the number of affected

individuals with type 2 diabetes worldwide at an estimated 347 million (WHO 2014 review) almost exceeding the predicted increase to 380 million by 2025 by Sicree *et al.* (2003). Zimmet *et al.* (2001) further stated that the prevalence of type 2 diabetes and obesity continues to increase in both developed and developing countries, thus presenting a major public health issue with various impacts on varying socio-economic issues.

Reports from the USA estimates that type 2 diabetes affects about 18.2 million people with data from studies done showing an increase in the prevalence amongst young people (Lin *et al.*, 2004). Also in aboriginal Canadians, type 2 diabetes mellitus has become an epidemic as the estimated prevalence in this population is 3-5 times higher than non-aboriginal Canadians (Ley *et al.*, 2009). In the study by Dyck *et al.* (2010), it was stated also that out of the 1500 first-nation people that were used for an experiment In Saskatchewan in 1937, diabetes was not detected among any of them. This statistic had changed by 1990, when research showed that almost 10% of the province's first-nation adults had diabetes, and this had increased in 2006, to over 20%, while it remained at about 6% in the general population in Canada (Dyke *et al* 2010).

These statistics beg the question of why the prevalence of diabetes appears to be on the increase world-wide. This therefore has led to studies into the possible risk factors of diabetes in order to prevent or delay the onset or properly manage the increase in the prevalence of diabetes. Traditionally some

of the risk factors that have been identified with diabetes are obesity, dyslipidemia, hyperglycemia and hypertension (Laakso 2010).

Overall, studies have shown that there is a link between obesity, cardiovascular diseases and diabetes (Barnes 2011). Obesity and diabetes mellitus are also suggested to be important independent conditions that may increase the risk of developing cardiovascular disease while obesity is the leading risk factor for Type 2 diabetes (Wang and Nakayama 2010). The Centers for Disease Control (CDC) in the United States of America further reported that the risk of developing diabetes in obese individuals is 93 times more than individuals with normal BMI, if the body mass index (BMI) is 35 kg/m. Data collected in the USA in 2007 showed that the national prevalence rates of diabetes had increased in parallel with the rates of obesity (Barnes 2011)

Previous studies also show that diabetes prevalence is on the increase in Africa, contrary to the belief in the last century that diabetes mellitus is rarely found in individuals in Africa. Studies carried out in Europe and America on African American, Afro-Caribbean subjects and African migrants in Europe showed that these populations had a high prevalence. African immigrants were reported to have higher prevalence and complications than the Caucasians living within the countries studied (Evaristo-neto *et al.*, 2010). In a study carried out in Angola, it was stated that the origin of this increase in prevalence may be

due to population growth, aging, urbanization, and increasing prevalence of obesity and physical inactivity (Evaristo-neto *et al.*, 2010).

Also, in the USA (Framingham in Massachusetts), a study by Hubert *et al.* in 1983 called The Framingham Heart Study (FHS), which was a longitudinal, multigenerational cohort study of obesity as the main predicting factor for the development of CVD. This study provided evidence which supports the fact that changes in the environment, behavior, and life-style of individuals has lead to a dramatic change in the incidence and prevalence of obesity in the volunteers and the corresponding increase in CVD. The Framingham study showed a similarity to statistics that came from the USA (Barnes *et al.*, 2011) and other developed countries (Shaw *et al.*, 2006) as it was noted that the prevalence of obesity (BMI $30 \text{ kg}/(\text{m})^2$) had risen (or is predicted to rise) and is associated not only with CVD, but also with diabetes..

The term diabetes mellitus is described by the WHO report of 1999 as a metabolic disorder characterized by chronic hyperglycemia (high blood sugar) which results from a deficiency in insulin production alone (type 1 DM) or in combination With a reduction in the sensitivity of target tissues to insulin. This report states further that the effects of this reduced availability of insulin results in disturbances in the metabolism of carbohydrate, fat and protein (WHO 1999).

1.2.1 Classification of diabetes mellitus

The fitting of an individual's diabetes mellitus into a particular classification is difficult as this depends on the circumstances of the individual which can make the person fall into more than one class at a time (for example a pregnant woman classified as having gestational diabetes can end up having type 2 diabetes after delivery)(American diabetes association (ADA 2008). Therefore, even though it's important to discuss the classification, it is also important to think of the classes in terms of their causes.

The earliest classifications of diabetes mellitus were described in the 1980 and 1985 classifications by the WHO expert committees. The 1980 classification included the classes IDDM (insulin dependent diabetes mellitus) or Type 1, and NIDDM (Non-insulin Dependent Diabetes Mellitus) or Type 2 (WHO 1999). However in the 1985 classification there was no mention of the Type1 or Type2 classifications but the classes IDDM and NIDDM were retained. The 1985 classification also included a class called the Malnutrition-related Diabetes Mellitus (MRDM) (WHO 1999)

In addition to these classifications both the 1980 and 1985 reports also included other types such as the Impaired Glucose Tolerance (IGT) as well as Gestational Diabetes Mellitus (GDM).The 1985 classification was widely

accepted and was used internationally because it allowed individual subjects and patients to be grouped in a more clinically useful manner even when the specific cause was unknown (WHO 1999).

It is however recommended that the ideal classification would encompass the clinical and etiological classifications (WHO 1999). The most recent classification by the American Diabetes Association recognizes the Type1, Type2, Gestational and the "other specific types" of diabetes (Genetic defects, Disease of exocrine pancreas, endocrinopathies, drug and chemical related gestational DM and infections related) (ADA 2008)

1.2.1.1 The clinical classification of diabetes mellitus

According to WHO (1999), diabetes mellitus can be clinically classified as diabetes requiring insulin for survival (equivalent to IDDM), those requiring insulin for control (equivalent to the NIDDM), and those that do not require insulin for blood glucose control as their blood glucose can be controlled with oral hypoglycaemic agents. It is further stated that clinically, the Impaired Glucose Tolerance (IGT) or the Impaired Fasting Glucose (IFG) are stages in the natural progression of the disorder in carbohydrate metabolism. However IGT have been used interchangeably with IGF but were shown not to be the

same conditions as IFG is a stage of derangement during fasting, while IGT is a stage after eating.

With regards to this, blood glucose was chosen as an objective measure to assess diabetes and the parameters used for IFG are; a fasting glucose concentration of 6.1 mmol/L (110 mg/dl) or greater (whole blood 5.6 mmol/L; 100 mg/dl), but less than 7.0 mmol/L (126 mg/dl) (whole blood 6.1 mmol/L ; 110 mg/dl) (WHO 1999).

When an Oral Glucose Tolerance Test (OGTT) (75mg of Glucose dissolved in 300mls of water is taken and blood glucose measured over 2 hours) is administered, some individuals with IFG can have results that suggest they have IGT or diabetes. Therefore whenever possible it is recommended that individuals with IFG have an OGTT to rule out Diabetes (Mayfield 1998).

Reference has also been made to a transit state of normoglycemia which is a clinical state where a patient has a "normal" glucose level with a fasting venous plasma glucose concentration of less than 6.1 mmol/l (110 mg/dl) (Mayfield 1998). This value is seen in individuals who have normal glucose tolerance but may have IGT on OGTT (WHO 1999). In this case it was suggested that the process that triggers diabetes starts when the individual has normal blood glucose.

1.2.1.2 The aetiological classification of diabetes mellitus

The aetiological classification is based on processes that may have led to the decline in the function of the insulin producing cells or the resistance of insulin target tissues to insulin (insulin resistance) (WHO 1999).

Seino *et al.* (2010) explains that aetiologically, type 1 DM is a type of diabetes that is due to the destruction of beta cells which reduces insulin in the blood due to inadequate amount of beta cells, thus making the individual dependent on insulin for survival. Without supplementing the insulin in these individuals, they become prone to comas, ketoacidosis or even death (ADA 2008). The presence of anti-GAD (antibodies to Glutamic Acid Decarboxylase), insulin and other islet cell antibodies suggest an auto-immune basis to Type 1 diabetes mellitus. In the paper produced by the WHO (1999), it was suggested that this auto-immune process may be slow in some and fast in others, thereby leading to an early and late onset in different individuals. When this occurs later than the usual onset, it is called the Latent Auto-immune Diabetes in Adults (LADA).

Type 1 diabetes is therefore highest in childhood or in adolescence but can occur at any age up to the 9th decade (WHO 1999) and the chances of the auto-immune destruction of the Beta cells are genetically pre-determined.

The WHO (1999) classification also includes the type 2 (T2DM) aetiological classifications which is the commonest and is characterized by poor insulin action and secretion. The etiology for the T2DM is unknown but is reported to be neither auto-immune nor caused by some specific causes of diabetes such as endocrinopathies, drug, infection and diseases of the exocrine pancreas. However many studies including that by Karam and McFarlane (2010) have shown that people with the type 2 diabetes are usually obese (BMI> 30). The risk of developing type 2 diabetes is also said to increase with age, obesity and lack of physical activity and said to be strongly associated with genetic links (WHO 1999).

Type 2 DM is different from Type 1 as it tends not to present with ketoacidosis except in severe infections and insulin levels may be found to be normal or high and may remain undiagnosed for many years as the high blood sugar is usually not high enough to provoke symptoms of diabetes (Mayfield 1998).

The reference in the present study to diabetes will be mainly to the type 2 diabetes as this is the most common (90%) (Stumvoll *et al.*, 2005) and reported to be most linked to obesity and cardiovascular diseases (Laarkso 2010) which are the areas this research is focused on.

Studies have shown that the early pathophysiology of type 2 diabetes is controversial and uncertain (ADA 2008) and the following sections explore further what previous researchers have proposed.

1.2.2 Structure and Chemical Properties of Insulin

Insulin is a polypeptide discovered in 1928 but it was not until 1952 that the amino acid sequence was discovered. It was reported that the amino acid sequence is arranged into two chains of different amino acid lengths which were called the A and B chains (Wilcox 2005). These chains together have 51 amino acids connected by disulphide bridges, with a molecular weight of 5802 and an iso-electric point of pH 5.5 (Please refer to figure 1.3).

Looking more closely at the structure of insulin, figure 1.3 shows that the A chain is made up of 21 amino acids with an N-terminal helix which is connected to an anti-parallel C-terminal helix on the B chain which has 30 amino acids. The two chains are connected by 2 disulphide bonds which connect the N and C terminal helices of the A chain to the central helix of the B chain. In pro-insulin, a connecting peptide links the N-terminus of the A chain to the C-terminus of the B chain (Wilcox 2005).

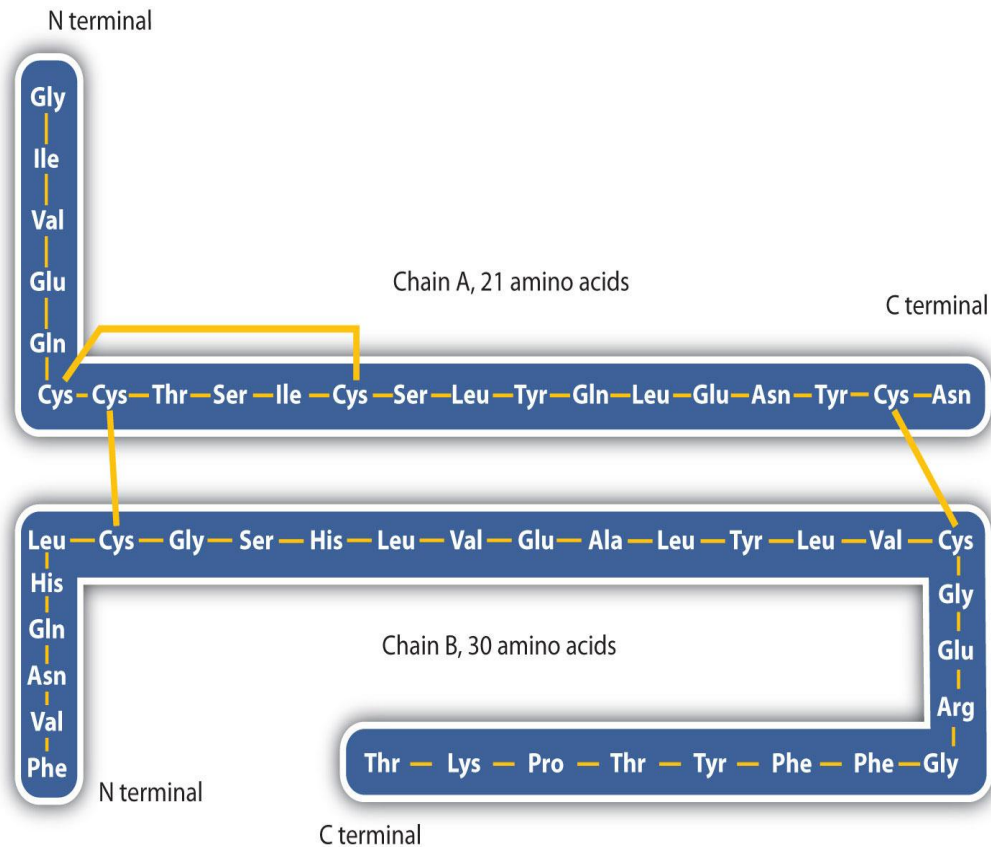


Figure 1.3 Figure showing the structure of the insulin. Chain A has 21 amino acids while chain B has 30. They both have a C and an N terminal that is linked by a disulphide bond (Modified from Ball *et al.*, 2011).

1.2.3 How insulin is synthesized and released

The gene that codes for insulin is found on the chromosome 11 (short arm) (Owerbach *et al.*, 1981). The process of synthesis of insulin begins by the

synthesis of its precursor (pro-insulin) in the β cells which are found in the pancreatic islets of Langerhans. Pro-insulin is first formed as pre-proinsulin in the ribosome of the rough endoplasmic reticulum (RER). Pre-proinsulin however, is synthesized by the formation of a protein which carries out signalling and which is linked to the B, C peptide and an A peptide chain. Pro-insulin is formed from pre-proinsulin by the removal of the protein which carries out the signalling from the pre-proinsulin (Wilcox 2005).

The pro-insulin that has been synthesized is then moved within a secretory vesicle from the rough endoplasmic reticulum to the Golgi apparatus. Due to the high concentration of zinc and calcium in the Golgi apparatus, there is the formation of pro-insulin molecules (hexamers) which contain zinc. The immature vesicles that are formed are acted on by the enzymes which act outside the Golgi therefore releasing insulin and C-peptide from proinsulin. Insulin therefore forms insoluble zinc containing hexamers which precipitate as crystals which are stable at a pH of 5.5 (Wilcox *et al.*, 2005).

Through exocytosis, equal amounts of insulin and C peptide are secreted within the mature granules into the portal vein in a pulsatile pattern (Wilcox *et al.*, 2005). Studies have also shown that the secretion of insulin in response to glucose stimulus occurs in 2 phases; the first being rapid and the second less rapid but more prolonged (Miele 2007).

1.2.3.1 Kinetics of Insulin Secretion

This can be described in stages and the first stage is triggered by a rise in blood sugar resulting in the secretion of insulin and C peptide from mature granules in the β cell. This process is usually mediated by a rise in the blood glucose but can be stimulated by other triggers (Rockl *et al.*, 2008). When the circulating blood glucose increases, the increase makes it possible for glucose to enter the beta cells where glucokinase phosphorylates glucose to glucose-6-phosphate using a molecule of adenosine triphosphate (ATP). Glucose-6-phosphate is then metabolized further by the TCA and glycolytic pathways leading to ATP production. As can be seen in figure 1.2, the production of ATP leads to an increase in the ATP/ADP ratio and the closure of K_{ATP} thereby leading to the depolarization and the activation of the voltage dependent calcium channels with an influx of calcium into the cells which triggers insulin secretion in a pulsatile manner (Miele 2007).

The second stage begins after the secretory granules have been refilled with insulin from the reserve pools. Acetylcholine (Ach) is then seen to stimulate phospholipase and protein C activation. Also this stage can be mediated with the activation of adenylyl cyclase activity, β cell protein kinase A, which encourages insulin secretion. The hormones that mediate the second phase are hormones such as vasoactive intestinal peptide (VIP), PACAP (Pituitary adenylyl cyclase activating polypeptide), GLP-1 (Glucagon-like peptide-1) and

GIP (Gastric inhibitory peptide) (these are referred to as “other signals” in figure 1.2) (Wilcox 2005).

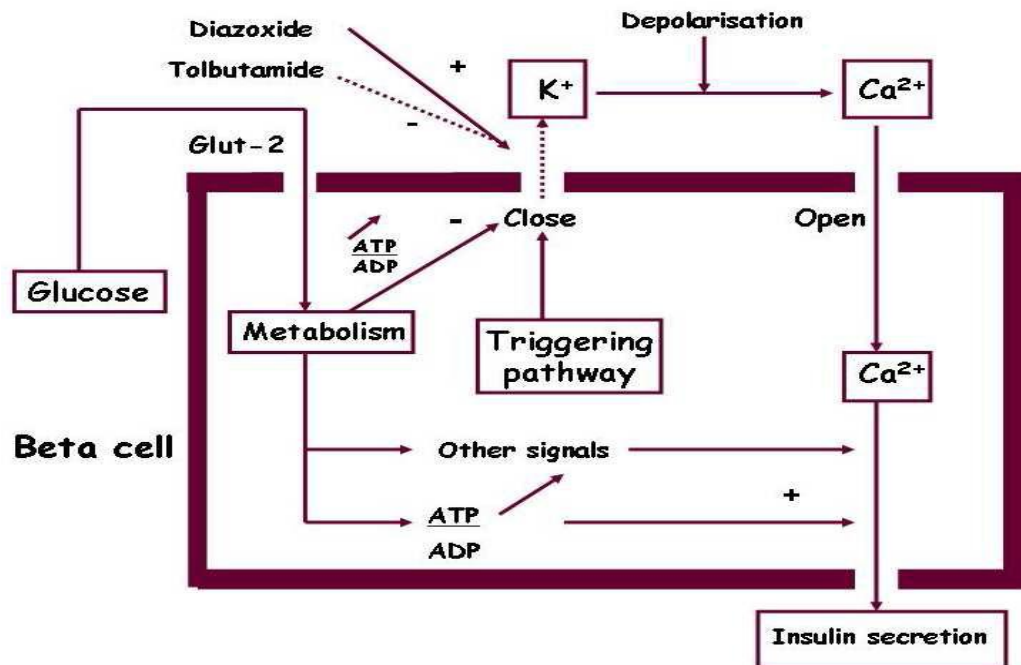


Figure 1.4 Figure showing the mechanism of insulin secretion from beta cells. The influx of glucose into the cells generate ATP which triggers a pathway that closes the potassium (K) channels on the cell membrane and therefore causes a depolarization which leads to the calcium channel (Ca) to open and an influx of calcium into the cells and a corresponding increase insulin secretion. (Modified from Henquin 2000)

1.2.4 Insulin signalling Pathway

After a meal, the carbohydrate component of food is broken down to glucose and this is used mainly in the muscles, adipose tissues and the liver. The

pathway that controls the movement of glucose between the blood and these tissues is called the insulin signalling pathway (Rockl *et al.*, 2008).

As seen in figure 1.5, the pathway begins when there is an increase in the blood glucose that supplies the pancreas which results in the beta cells of the pancreas releasing insulin into the blood stream and the insulin is carried to the tissues where they will be used after binding to the insulin receptors in these tissues. The insulin receptor consists of two α subunits and two β subunits that are disulfides linked into a $\alpha_2\beta_2$ heterotetrameric complex. The α subunit are extracellular and insulin binds to these first. The binding of insulin sends a signal that activates the tyrosine kinase unit of the β unit which causes intramolecular transphosphorylation in which the β subunit causes phosphorylation of adjacent subunits in their tyrosine residues (Saltiel and Kahn 2001).

The binding of insulin to the receptor increases the affinity of the receptor for a protein called insulin receptor substrate 1(IRS-1). The receptor is also responsible for the phosphorylation of IRS-1. The phosphorylated IRS-1 then phosphorylates phosphatidyl inositol-3 (PI-3) kinase which therefore phosphorylates phosphatidyl inositol diphosphate (PIP2) by adding a phosphate group to it to form phosphatidyl inositol triphosphate (PIP3). This extra phosphate group added creates a binding site for PDK1 which when phosphorylated helps to phosphorylate AKT (Stumvoll *et al.*, 2005).

AKT (also called protein kinase B-PKB) is the kinase that phosphorylates GLUT-4 (Glucose transport protein-4) and helps its translocation to the membrane of the cells where it is anchored to the cell membrane and allows the diffusion of glucose from the blood into the cell via tubules on the GLUT-4 (Wilcox 2005)

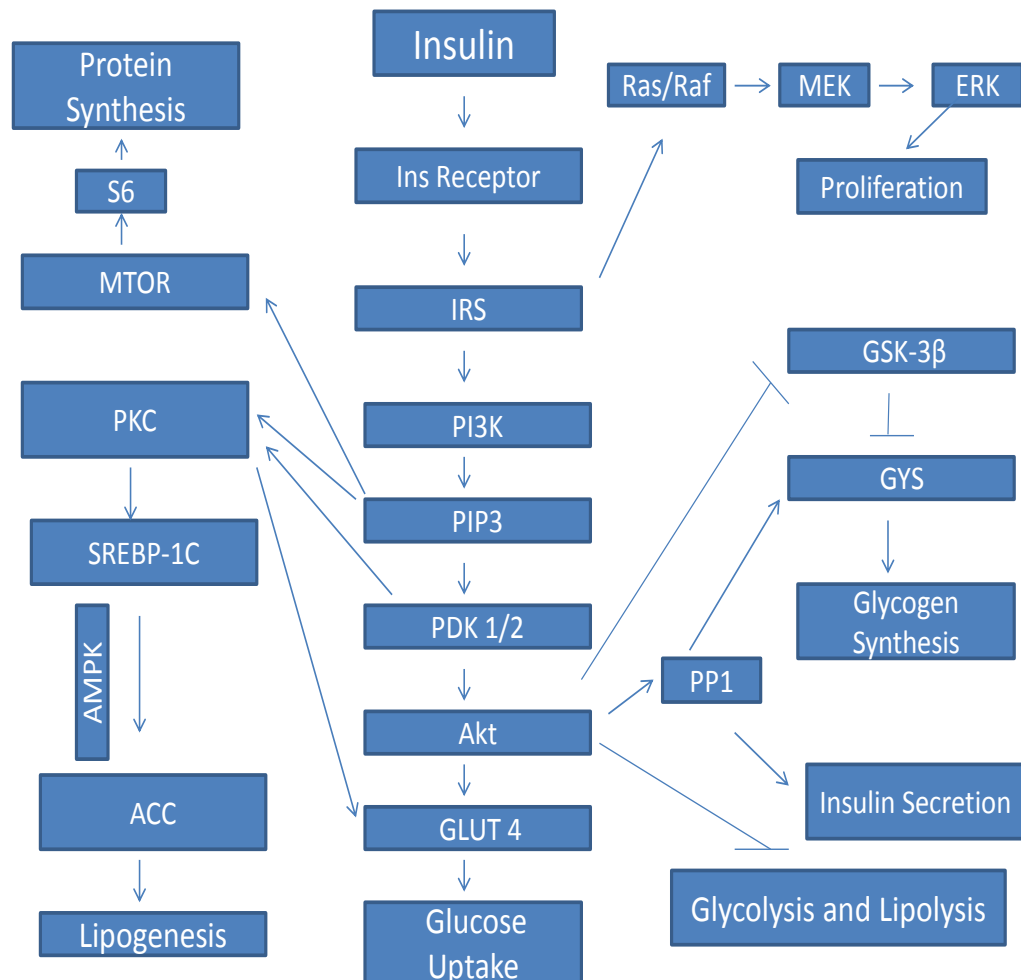


Figure 1.5 Figure showing the insulin signalling pathway. Insulin goes through IRS, PI3K, Akt to influence GLUT4 which translocates to the cell membrane and allows glucose to go into cells. (Modified from Kanehisa laboratories 2013).

1.2.4.1 Insulin Receptors and Insulin Binding

In order to carry out its effects, insulin binds to a receptor which consist of 2 α and 2 β glycoprotein subunits which are found on the cell membrane and are connected by disulphide bonds (Saltiel and Kahn 2001).

The gene that codes for the insulin receptor is found on the short arm of chromosome 19 (Wilcox 2005) and once insulin binds to the α subunit, the effect inhibits the kinase activity in the β subunit leading to a conformational change which makes it possible for ATP to bind the intracellular β subunit part of the receptor and carry out phosphorylation (Wilcox 2005). The binding of ATP stimulates the phosphorylation of the β subunit which gives it tyrosine kinase activity. This is followed by tyrosine kinase phosphorylation of intracellular cell proteins called Insulin Responsive Substrates (IRS). The IRS then binds other molecules that mediate various signal pathways which mediate the function of insulin (Hotamismigil *et al.*, 1996).

The insulin receptor substrate (IRS) has been classified into 4 types; IRS 1,2,3,4 (Saltiel and Khan 2001). IRS 1 is the substrate responsible for the mitogenic effects of insulin in skeletal muscles and acts as a link between the sensing of glucose and insulin secretion. In order to function, IRS 1 needs to be

phosphorylated by the insulin receptor and insulin-like growth factor 1 receptor (IGF-1) (Wilcox 2005).

The IRS 2 helps to mediate the actions of insulin at target sites and also mediates the growth of pancreatic β cells. The IRS 2 is mainly found in the liver (Wilcox 2009). IRS 3 is found mainly in the adipose tissue, pancreatic β cells and liver while the IRS 4 is majorly found in the thymus, brain and kidney (Saltiel and Kahn 2001). The function of IRS 3 and 4 are not well defined (Wilcox 2005).

The pathway by which insulin influences metabolism in the human body involves the use of IRS. Insulin stimulates the receptors which use IRS as substrates within the cell and the binding of the phosphorylated IRS to phosphatidylinositol-3 kinase (PI-3K) (which acts on Akt and protein kinase B (PKB) leading to the translocation of the glucose transporter proteins (GLUT4) to the cell wall) allows the uptake of glucose into the cells. Also phosphorylated IRS bound to PI-3 kinase acts on protein kinase C (PKC) and PI dependent protein kinases 1 and 2 (PIPK 1 and 2) leading to the conversion of glucose to glycogen and also prevents the breakdown of lipid and helps to synthesize protein and the regulation of hepatic gluconeogenesis (Wilcox 2005).

Insulin functions mainly in the mediation of the uptake of glucose by various tissues but it also encourages the uptake of other food nutrients such as protein and fat. After a meal, the glucose level in the vein that leaves the intestine is elevated as there is absorption of glucose. A third of this glucose is absorbed into the liver and the rest is used for the metabolic function of the body. The rise in blood sugar leads to a corresponding increase in insulin synthesis by the pancreas. The presence of insulin therefore makes it possible for skeletal muscles to make use of glucose. Insulin influences the metabolism of carbohydrate, lipid, and protein and is seen to influence the transcription and translation process of the messenger ribonucleic acid (mRNA) (Wilcox 2005).

1.2.4.2 Insulin Receptor substrate-1 (IRS)-1 and 2

The first major receptor substrate for insulin receptors and the IGF-1 (Insulin-like growth factor 1) receptor tyrosine kinases to be identified is the Insulin Receptor Substrate (IRS)-1. In the earlier report by Sun *et al.* in 1991, it was reported that insulin carries out its function by binding to the alpha subunit of the insulin receptor, resulting to the stimulation of the beta subunit of the receptor to cause a tyrosine phosphorylation of the proteins within the cells. The first protein that was discovered was called the Insulin Receptor Substrate 1 (IRS-1).

Other studies carried out on cells have shown that many of the responses to the effect of insulin are mediated by counter-regulatory hormones and pro-inflammatory cytokines via the effect of Tyrosine and Serine which phosphorylates insulin receptor substrate 1 and 2. (IRS-1,2) (Lin *et al.*, 2004)

Glucose tolerance was also noted to have been impaired by the dysregulation of these IRS proteins by pro-inflammatory cytokines or genetic deletion which leads to insulin resistance (Lin *et al.*, 2004). Furthermore, the deficiency of IRS-2 was found to lead to life threatening type-2 diabetes due to its role in cell growth, function and survival. This is further evidenced by the fact that the progression to a state of type-2 diabetes was found to be prevented by modifying elements of the insulin/IGF-signaling cascade that promote compensatory cell function (Lin *et al.*, 2004).

Kubota *et al.* (2000) investigated the role of the IRS in the execution of insulin's function and it was discovered that homozygous IRS-1 deficient mice were deficient in embryonic and post natal growth, therefore suggesting that the IRS-1 is critical in the growth promoting function seen in insulin and IGF. Also in this study, resistance to the effect of insulin and IGF in lowering blood glucose was suggested as a basis for insulin resistance in diabetes. In spite of all these, it was realised that the mice maintained a normal fasting glucose which is ascribed to compensatory beta cell hyperplasia. It was therefore suggested that the signal transduction in insulin and IGF can be IRS-1 dependent and also be

IRS-1 independent. This IRS-1 independent transduction pathway was later identified to be mediated by an alternative substrate in the liver which was named IRS-2 by Kahn's group (Kubota *et al.*, 2000).

Another line of research uncovered a protein called pp-190 which is a protein with molecular weight of 190kDa protein first discovered in a study on rats which were IRS deficient. It was reported to be located in the liver of the rats and has now been identified as IRS-2 (Lin *et al* 2004). It was suggested that this protein is responsible for the tyrosine phosphorylation by insulin and that this molecule has the capacity to bind both 85-kDa subunit of phosphatidylinositol (PI) 3-kinase (Kubota *et al.*, 2000). In the study by Kubota *et al.* (2000) it was also noted that there was a corresponding amount of tyrosine phosphorylation of IRS-2 in the liver of IRS-1 deficient mice when compared to tyrosine phosphorylation of IRS-1 seen in wild-type mice thereby compensating fully for the effect of IRS-1 deficiency in the normal actions of insulin. Furthermore, the activation of PI 3-kinase stimulated by insulin was noted to be normal in the liver of IRS-1 deficient mice which had a defective PI-3 activation in the skeletal muscle. This was attributed to a reduction in the tyrosine phosphorylation of IRS-2 compared with IRS-1 in wild type mice further suggesting the possible importance of IRS-2 in the functions of insulin especially in the liver (Lin *et al.*, 2004).

1.2.4.3 Protein phosphatase (PP-1) expression in beta cells

Figure 1.1 shows that leptin binds to its receptor and activates the ObR-associated JAK2 tyrosine kinase via transphosphorylation. This process phosphorylates tyrosine residues of ObR and the phosphorylated STATs dimerize and translocate to the nucleus to regulate gene transcription (Marroqui *et al.*, 2012). STAT is said to affect the expression of the catalytic subunit Ppp1ca of PP-1 due to the influence of leptin signalling resulting in a decrease in the expression of PP-1 gene which leads to reduced PP-1 enzyme activity and a subsequent reduction of intracellular calcium concentrations (Seufert 2004). Previous research has shown that insulin secretion is inhibited by reduced intracellular calcium in pancreatic cells (Saltiel and Kahn 2001). Therefore the suggestion in the report by Kuehnen *et al.* (2011) that leptin reduces the generation of the mRNA for the Protein phosphatase (PP-1) gene and the protein that it codes for can be explained by the fact that the insulin secretion stimulated by glucose is inhibited by nuclear inhibitor of PP-1, which subsequently leads to reduced PP1 in the cytosol and then a reduction of calcium influx which is normally stimulated by the presence of PP-1. Therefore in the present study, beta cells will be treated with a range of concentrations of leptin at various glucose levels. The cells will be collected, mRNA extracted from them and reverse transcription PCR done to assess the effect of leptin on the expression of PP1A gene.

1.2.5 Insulin and the uptake and use of nutrients

1.2.5.1 Insulin and the uptake and use of carbohydrate

The process of carbohydrate metabolism is highly influenced by the effect of insulin. Insulin helps to move glucose into fat and muscle cells as a result of its effect on GLUT 4 translocation (Saltiel and Kahn2001).

As can be seen in figure 1.5, insulin facilitates the synthesis and storage of glycogen by increasing the uptake of glucose into the liver and the mediation of glycogen synthesis in the body. The accumulation of glycogen starts when insulin stimulates the dephosphorylation of the glycogen synthetase by inhibiting kinases such as protein kinase A (PKA) and the stimulation of protein phosphatase 1 (leptin inhibits this and therefore leads to the inhibition of glycogen synthesis) (Saltiel and Kahn 2001). This further leads to activation of Akt and a reduction of the phosphorylation of glycogen synthetase and an increase in its activity.

In the liver, however, insulin blocks gluconeogenesis and glycogenolysis thereby causing an inhibition of glucose synthesis in the liver. Insulin is said to have its influence in the body by either phosphorylation or dephosphorylation or the regulation of genes that code for some of the substrates and enzymes

which are responsible for glycolysis and gluconeogenesis. Insulin is able to inhibit gluconeogenesis by inhibiting the gene that codes for phosphoenolpyruvate carboxylase which is the enzyme responsible for carrying out the most critical step in gluconeogenesis. (Saltiel and Kahn 2001)

When there is need for energy, however, insulin causes pyruvate kinase dephosphorylation leading to the stimulation of glycolysis and the inhibition of gluconeogenesis by the dephosphorylation of 2,6 biphosphate kinase, thus making more glucose available for use to generate energy. Also, intracellular energy abundance results in the activation by insulin, of intra-mitochondrial pyruvate dehydrogenase. This however leads to an irreversible conversion of pyruvate to acetyl co-A, which is oxidised by the Krebs cycle in the synthesis of fatty acid (Wilcox 2005).

1.2.5.2 Insulin and the use and uptake of lipids and fat

Insulin helps to mediate the uptake and utilization of lipid and fat in various target tissues. Some of these tissues are the adipose tissue, the liver and lactating mammary glands. Insulin also helps in the formation and storage of triglycerides in adipose tissue (Wilcox 2005). Just like its effect on carbohydrate, it encourages the production of lipids and reduces their breakdown.

Insulin mediates the synthesis of triglycerides via a process which involves the esterification of glycerol phosphate, but the suppression of the breakdown of fat by oxidation occurs when insulin inhibits carnitine acyl transferase and also via the dephosphorylation of the hormone sensitive lipase which helps to break down triglycerides (Wilcox 2005). Insulin inhibits the activity of the lipase primarily through reductions in cAMP levels, owing to the activation of a cAMP-specific phosphodiesterase in fat cells (Saltiel and Kahn 2001).

An alternative method for the control of metabolism of lipids by insulin is seen when we consider the effect of insulin on carbohydrate metabolism. Therefore in the fat cells, when there is an excess of glucose, insulin mediates the conversion to lipids via the activation of lipid synthetic enzymes, including pyruvate dehydrogenase, fatty acid synthase and acetyl-CoA carboxylase (ACC as seen in figure 1.5) (Saltiel and Kahn 2001).

Insulin also regulates the uptake and metabolism of cholesterol by activating and dephosphorylation and activation of HMG Co-A reductase. The breakdown of cholesterol ester is however inhibited by the inhibition of cholesterol esterase by insulin (Wilcox 2005).

1.2.5.3 Insulin and the uptake and use of protein

Insulin functions as a mediator in the synthesis of protein. The protein synthesis action of insulin is seen in many target tissues and is intracellular in nature. Furthermore, insulin helps in the protein synthesis process by transcription of specific mRNA and the translation of these mRNA into protein intracellularly, a process which is carried out in the ribosomes. Examples include mRNA transcription for fatty acid synthetase protein kinases and glucokinase (Wilcox 2005).

Protein is made up of amino acids and the amount of protein in the body is a reflection of the amount of amino acids present. The total amount of amino acids left in the body is a balance between the amount taken in and the amount removed and these processes are influenced by the use in protein synthesis, amino acids that come out of tissues after tissue breakdown and oxidation in the body. The effect of insulin on protein metabolism involves a reduction in plasma amino acids, an effect which is most marked for the branched-chain amino acids (BCAA)' and minimal for alanine (Fukagawa *et al.*, 1985).

The effect of insulin on transcription is also seen in albumin synthesis which occurs in the liver, the pyruvate carboxylase process in the adipose tissues, casein synthesis in the mammary glands and amylase in the pancreas.

However, insulin action may reduce mRNA synthesis for enzymes in the liver such as carbomyl phosphate synthetase which is one of the enzymes that influences the urea cycle (Wilcox 2005). It was also stated by Wilcox (2005) that insulins' effects on translation are seen in many tissues and insulin works by itself and in partnership with growth factors such as Insulin Growth Factor 1.

1.2.6 Role of insulin in energy homeostasis.

Most cells in the body utilize glucose for metabolism. This therefore suggests how useful insulin is as a result of the fact that insulin is very important in mediating the regulation of cellular energy and macronutrient balance. With regards to metabolism, insulin helps to maintain homeostasis of energy by suppressing the breakdown of adipose tissue when there is abundance of energy and allowing glucose to enter cells and in the muscles, the entry of glucose into the cells enables the synthesis and storage of glycogen. Glycogen forms a reserve of energy for the body when it is in a condition where the immediate energy supply is inadequate (fasting or exercise). If in this case the glycogen store is used up and there is still no supply of energy, then the body uses amino acids or fatty acids (Stumvoll *et al.*, 2005).

1.2.7 Pathophysiology of diabetes mellitus

1.2.7.1 Genetic basis of diabetes mellitus

The process of developing type 2 diabetes is typified by 2 main factors; resistance to insulin in tissues like muscle and liver and also a dysfunction of insulin secretion. Previous researchers have however not been able to find out if only one genetic defect causes the dysfunction in insulin function and secretion at the same time, in the common forms of human diabetes. Studies have reported that type 2 DM is polygenic (Sacks and McDonald 1996). This has led to further work being done on the genetic and molecular basis of diabetes (Vigliotta *et al.*, 2004).

Data gathered over the years have suggested that type2 DM has a genetic link. Studies have shown that the presence of a family history gives a 2-4 fold increase in the chances of developing type 2 DM. Also it has been reported that 15-25% of the individuals who are first-degree relatives of patients with type 2 DM may develop impaired glucose tolerance or diabetes. Also, it was suggested that the lifetime chance of developing T2DM at the age of 80 years is about 38% if there is a history of one parent having T2DM. Also it has been shown that there is a 60% chance of developing T2DM at age 60yrs, in offsprings of patients with T2DM, if both parents are affected (Stumvoll *et al.*, 2005).

Stumvoll *et al.* (2005) also suggested that the result of research on twins showed that concordance rates for type 2 DM were 35-58% in monozygotic compared to 17-20% in dizygotic individuals older than 60 years, and that when impaired glucose tolerance was considered, concordance in monozygotic twins was markedly increased to 88%.

Recent research has suggested the involvement of PED/PEA-15 which is a mitogen signaling protein and found to be increased in diabetic patients. It was suggested that PED/PEA-15 expression leads to the inhibition of the transport of GLUT 4 to the membrane for glucose transport thereby leading to insulin resistance. Also, insulin receptor substrate 1 (IRS-1) and protein phosphate 1(PP-1) gene expression have been suggested to be involved as stated in the appropriate sections above (Vigliotta *et al* 2004).

1.2.7.2 Insulin derangements as a basis of diabetes mellitus

Insulin is described by Wilcox (2005) to be a protein hormone which maintains normal blood glucose levels by mediating cellular glucose uptake, the metabolism of carbohydrate, lipid and protein metabolism, and promoting cell division and growth through its mitogenic effects. Insulin is secreted by the β cells of the islets of Langerhans in the pancreas.

1.2.7.3 Insulin Resistance

Insulin resistance is a condition in which the insulin mediated glucose uptake effect seen is less than that expected for glucose uptake in skeletal muscles and also for the suppression of glucose production within the liver (Strumvoll *et al.*, 2004). Insulin is responsible for the uptake of glucose in the skeletal muscles and the suppression of glucose synthesis by the liver and fatty acids by adipose tissue. In type 2 diabetes, endogenous glucose production and fatty acid production are increased, while there is a reduction of glucose uptake by the skeletal muscle. Due to an increase in blood glucose despite hyperinsulinemia, especially in the early stages of T2DM and in impaired fasting glucose, the resistance to insulin in the liver is reported to be responsible for hyperglycemia in T2DM (Strumvoll *et al.*, 2004). The greatest effect of insulin resistance is seen in the skeletal muscle and most tissues in the periphery. Insulin resistance typifies the type 2 diabetes mellitus (Wilcox 2005).

Insulin is the main hormone which affects many anabolic processes in the body and together with other hormones such as growth hormone and Insulin-like Growth Factor (IGF-1), insulin is able to effectively regulate the metabolic processes in the human body. Growth hormone which is responsible for growth is secreted as a result of the stimulus by insulin when there is a high level of insulin to stop insulin from reducing glucose below the normal level, thereby ensuring a steady level of blood sugar (Wilcox 2005).

There are other hormones that oppose the effect of insulin some of which are glucagon, glucocorticoids and catecholamines. These counter-regulatory hormones support metabolism in the body in the fasting condition as seen in the action of glucagon in glycogenolysis, gluconeogenesis and ketogenesis in fasting states. Catecholamines may also encourage lipolysis while glucocorticoids encourage glycogenolysis, lipolysis and catabolism. When these counter-regulatory hormones are secreted in excess they may lead to insulin resistance, but this process is not thought to be responsible for most of the insulin resistance states (Wilcox 2005). Further studies have shown that the resistance to insulin occurs at the level of the cells and that the mechanism responsible for insulin resistance at the cellular level is one of post-receptor defects in insulin signalling. Some of the mechanisms include the down-regulation of receptors, lipotoxicity, gene deficiencies or genetic polymorphisms affecting tyrosine phosphorylation of the receptors for insulin sensitivity, IRS proteins or kinases of PIP-3 (Phosphatidylinositol (3,4,5)-trisphosphate) and derangements of GLUT 4 function (Abdul-Ghani *et al.*, 2007, Abdul-Ghani and DeFronzo 2009, Wilcox 2005).

Insulin influences the metabolism of various tissues and as the effect of insulin varies with the different tissue function. Furthermore, the mechanisms responsible for insulin resistance in target tissues also vary. In order to shed more light on this, the mechanism and effect of insulin resistance has been investigated and will be discussed in the following sections.

1.3 Cardiovascular diseases

Cardiovascular disease is defined by Fuster and Kelly (2010) as a condition that is made up of cardiac disease, vascular diseases of the brain and kidney, and peripheral vascular disease.

1.3.1 Epidemiology of cardiovascular Diseases

Cardiovascular disease has been suggested to be a problem of the very developed nations but recent data from the WHO in 2008 (WHO 2008) shows that nearly 30 percent of all deaths in low and middle income countries are attributable to CVD, and more than 80 percent of CVD-related deaths worldwide now occur in low and middle income countries.

Studies have shown that by 2005, the total number of cardiovascular disease (CVD) deaths (mainly coronary heart disease, stroke, and rheumatic heart disease) had increased globally to 17.5 million from 14.4 million in 1990 (Fuster and Kelly 2010). When considered closely, coronary artery disease was found to be responsible for, 7.6 million while stroke accounted for 5.7 million. Further studies have shown that of these deaths, more than 80 percent of the deaths occurred in low and middle income countries. Considering these statistics, the

World Health Organization (WHO 2008) estimates there will be about 20 million CVD deaths in 2015, accounting for about 30 percent of all deaths worldwide (WHO, 2005). Projections made, based on the trends, suggest that CVD mortality, will over the next few decades be responsible for more deaths than infectious and other chronic diseases, and that by 2030, diseases that are communicable will account for less than one-quarter of the mortalities worldwide (Fuster and Kelly 2010). Thus, CVD is expected to be the highest contributor to global mortality (WHO, 2009b).

Turning to Europe it is estimated that cardiovascular diseases contribute to about 40% of the mortality rate for the population across all causes. When the various causes of CVD are considered, coronary heart disease and stroke were suggested to be the most important (Khromhout 2001). Data collected from previous studies shows that coronary heart disease contributes about 50% to total cardiovascular diseases and stroke about 25% in Europe (Khromhout 2001). Data from the USA suggest that CVD was responsible for a mortality of 36% of all deaths in 2004.

1.3.2 Pathophysiology of cardiovascular diseases in the blood Vessels

Data from previous studies have shown that about 45% of the cases of CVD were due to coronary heart disease while about 30% were due to Stroke (Fuster and Kelly 2010). This suggest that majority of the pathology in CVD is in the arteries. The arteries are lined by endothelial cells and one of the pathogenic factors in CVD is endothelial cell dysfunction which has various pathways (Libby and Theroux 2005).

Previous studies have looked into and have described the normal function of the endothelial cells. It was suggested that the endothelium maintains the normal homeostasis of the vessel by producing extracellular matrix components like collagen, mediators of regulatory processes such as nitric oxide (NO), , endothelin-1 (ET-1),) Von Willebrand factor (VWF) angiotensin II (ANG-II), tissue-type plasminogen activator (t-PA), plasminogen activator inhibitor-1 (PAI-1), prostanoids (prostacycline, adhesion molecules and cytokines, the most important of which is Tumor Necrosis Factor (TNF)(Ambrose and Barua 2004 , Barrett et al 2010).

In its role in sustaining the integrity of the vascular wall, fibrinolysis is controlled by the endothelium when it produces tissue plasminogen activator (t-PA) and

Plasminogen activator inhibitor 1 (PAI-1) which inhibits it. The endothelium also influences the function of heparin sulphate/antithrombin, thrombomodulin/protein C and tissue factor/tissue factor inhibitor interactions, which limit the coagulation cascade (Barrett *et al.*, 2010). In addition to this the endothelium influences vascular remodelling by a process that involves the release of factors that promote or inhibit the growth and differentiation of the blood vessels such as TIMPs and MMPs, platelet-derived growth factor (PDGF) and angiotensin II (ANG II). The repair and maintenance of the extracellular matrix in the blood vessels is dependent on a balance between MMPs which break down the ECM and the TIMP that inhibits MMPs (Libby and Theroux 2005).

Any deviations in the normal functions of the endothelial cells, therefore leads to cardiovascular diseases. In normal physiological situations the production of factors that relaxes the endothelium such as nitric oxide (NO) and prostacyclin (PGI₂), and factors that mediate contraction such as endothelin-1 (ET-1), prostaglandins, and angiotensin II (ANG-II) are held at a balance which may be affected by risk factors for CVD, thus initiating the process that may result in atherosclerosis (Feldman *et al.*, 2000).

There are factors that prevent inflammation in the blood vessels. NO (Nitric Oxide) inhibits the adhesion of leucocytes as well as VCAM expression that is cytokine-induced. NO carries out the anti-inflammatory function by the inhibition

of transcription factor nuclear factor κ B (NF- κ B). NO production is diminished in cells which are subject to oxidative stress. When there is an increase in oxidant generation, a failure to repair oxidative damage or a decrease in antioxidant production, there is oxidative stress. The cause of the cells death is said to be a reactive oxygen species (ROS). When there is oxidative stress, cells that scavenge for damaged cells and mediate inflammation are not able to function due to the amount of ROS. Some of the risk factors that have been linked with oxidative stress are insulin resistance, dyslipidemia, inflammation, and also cigarette smoking (Stapleton *et al.*, 2008).

When insulin binds to the insulin receptor, it stimulates the phosphatidyl-inositol pathway (PI-3) which results in the activation of Akt which stimulates both the GLUT-4 activation and uptake of glucose in skeletal muscles and the eNOS endothelial pathway which stimulates the production of Nitrogen Oxide (NO) that is responsible for mediating the relaxation of blood vessels. The other half of the insulin signalling pathway also influences endothelial cells via the Shc/Ras/ mitogen-activated protein (MAP) kinase pathway which leads to the synthesis of endothelin-1 (causes vasoconstriction) and VCAM which encourages smooth muscle migration and mitogenesis in endothelium leading to angiogenesis (Laakso 2010).

When there is a pathway-specific impairment in the PI 3-kinase dependent pathway, insults such as proinflammatory cytokines (tumor necrosis Factor

TNF, interleukin (IL)-1, IL-6, and other proteins such as C-reactive protein (CRP)), which may cause an imbalance between production of NO and glucose uptake in the endothelium and, this may result in insulin resistance and endothelial dysfunction. The resulting hyperglycemia further leads to an inhibition of NO production. The reduction in NO production together with an elevation of FFA levels due to impairment in insulin's antilipolytic effect causes an increase in the reactive specie production which further contributes to the decrease in the synthesis of NOs (Laarkso 2010). Hyperglycemia has also been linked to an increase in the production of endothelin -1 which leads to vasoconstriction. As a result of all these processes mentioned, it has been suggested that abnormal smooth muscle cell function may be caused by diabetes as a result of an impairment of NO mediated vasodilation, increased levels of endothelin-1, angiotensin II, and plasminogen activator inhibitor 1 (PAI-1). This suggests that hyperglycemia causes a further down-regulation of the PI 3-kinase pathway and that there is further up-regulation of the Shc/Ras/MAP-kinase in the pre-diabetic state, thus demonstrating diabetes as a pathway for the development of CVD (Strienstra *et al.*, 2007).

1.3.3 Specific genes of interest in atherosclerosis and angiogenesis

Angiogenesis and atherosclerosis account for most of the pathogenesis of CVD (Lip 2004). Angiogenesis which is the formation of new blood vessels is linked

to atherosclerosis (formation of an atheromatous plaque in the blood vessels or heart) and both form most of the basis for CVD (Nagase *et al.*, 2006). Previous studies suggest that CVD occurs when there is a derangement in the balance in the remodelling process carried out by matrix metalloproteinases (MMP) which break down excess tissues formed when blood vessels are repaired and the tissue inhibitor of metalloproteinases (TIMPs) which inhibit MMPs, thereby ensuring just enough tissue formation in injuries and inflammatory processes within the blood vessels (can also lead to excess accumulation of collagen or extracellular matrix if there is an expression in higher than normal amounts (Hemmann *et al.*, 2007). It is important to note that TIMP in practical terms act as a balance to MMPs and TIMP can predispose to CVD (atherosclerosis) if there is over-expression without a corresponding amount of MMPs to inhibit, while the MMPs can result in angiogenesis and excess expression can lead to weakening of blood vessels, and over stimulation of the migration of smooth muscle cells and macrophage activation leading to an atheromatous plaque (Dollery *et al.*, 1995).

There are 3 angiogenic genes of interest which are MMP2, TIMP2, and TIMP3. MMP2 is a metalloproteinases that is found on the endothelial cell surface and carries out the function of extracellular matrix turnover by regulating the cell-ECM interactions (Park *et al.*, 2001). TIMP2 binds to it to form a ternary complex thereby inhibiting its function in the body (Stetler-Stevenson *et al.*, 1989). Metabolic syndrome has been associated with increased levels of expression of MMP2 genes (Hopps and Caimi, 2012). Studies have shown

raised levels of MMP2 in obese rats in contrast to some studies that have shown lower levels of MMP2 in obese individuals than lean (human). Diabetics are reported to express MMP2 more in the endothelial cells and monocytes in high glucose situations. Also, a higher MMP2 activity has been found in hypertensive patients and in dyslipidemic patients (Hopps and Caimi, 2012). MMP2 is reported to be linked with cardiac rupture and aortic aneurysms as it is said to break down elastin, type IV collagen and other ECM molecules (digests collagen I, II, III and intracellularly digest troponin, myosin light chain and poly (ADP-ribose) polymerase in cardiac cells (Nagase *et al.*, 2006). MMP2 gene expression is said to be inhibited by adiponectin and expression increased by leptin (Madani *et al.*, 2006).

TIMP2 inhibits the actions of MMP2 and leptin increases the expression of TIMP2 in endothelial cells (Park *et al.*, 2001). TIMP2 is suggested to be linked to human obesity and diabetes complications. It is said to suppress the multiplication of endothelial cells by inducing gene expression which promotes the G (1) arrest in the cell cycle and inhibits cell migration (Minchenko *et al.* 2013). Leptin has been found to increase the expression of TIMP2 (Park *et al.*, 2004) while Chen *et al.* (2012) and Kim *et al.* (2009) suggests that adiponectin increases TIMP2 gene expression.

TIMP3 is thought to be the only metalloproteinase inhibitor that targets the effect of MMPs in the extracellular matrix (ECM) and it was suggested that a

polymorphism of TIMP3 is associated with hypertension and a lack of TIMP3 leads to suppressed hypertensive response to angiotensin II (Basu *et al.*, 2013).

Furthermore, it is important to look at the other basis of the pathogenesis of CVD which is atherosclerosis. Atherosclerosis is a process that involves formation of an atheromatous plaque which results from injuries to the vasculature lining. This injury causes the release of cytokines that attract platelets, monocytes (which LPA converts to foam cells), smooth muscle cells and endothelial cells (influenced by TIMP) and fats cells (influenced by LPL). Therefore angiogenesis also contributes to atherosclerosis.

Lipoprotein lipase (LPL) can be produced from the vessel wall (macrophages within plaques) or from the plasma (from adipose cells and muscles cells). The vessel wall LPL is reported to cause an increase in the risk of atherosclerosis while the plasma LPL decreases the risk of atherosclerosis (Mead and Ramji., 2002). Also, LPL is reported to promote the retention of lipoproteins in the sub-endothelial space thereby favouring the adhesion of monocytes to the endothelium and it is also reported to mediate the conversion of macrophages into foam cells and to stimulate the process that leads to production of proinflammatory cytokines, and the proliferation of smooth muscle cells which all predispose to atherosclerosis (Maingrette and Renier 2003). Leptin is thought to encourage atherosclerosis by increasing the effect of the vessel wall LPL from macrophages. Most of the previous studies were unable to show the

expression of the LPL gene in endothelial cells and have suggested that the tissues that are responsible for the synthesis of LPL are the adipocytes, muscles cells, liver cells or macrophages (Hirata *et al.*, 1999).

Lipoprotein-a (LPA) is a protein molecule made up of LDA (an LDL like protein) and apolipoprotein. LPA is said to be pro-atherosclerotic because it causes accumulation of fibrin as a result of the inhibition of the activation of plasminogen to plasmin (which breaks down fibrin in blood clots), thus leading to the accumulation of blood clots (Buechler *et al.*, 2001).

1.3.4 Pathophysiology of CVD in the heart

The tumour necrosis factor (TNF) is an important cytokine in the process of developing CVD in the heart. In its normal function, some researchers have shown that TNF-alpha can have a paradoxical effect as it can stimulate tissue building by increasing fibroblast and mesenchymal cell proliferation and the stimulation of growth factors such as vascular endothelial growth factor (VEGF) (Trayhurn and Wood 2004). On the other hand TNF-alpha can also cause the destruction of tissues of the heart by the release of collagenases such as MMPs and can cause the inhibition of the synthesis of their inhibitors (TIMPs). TNF alpha when expressed in large amounts greater than the TNF receptors available, as seen in inflammatory conditions, can result in the destruction of

heart tissues and hypertension which may eventually lead to damage to the heart by increasing the adhesion molecule expression and increasing scavenger receptor class A and the uptake of oxidized LDL by macrophages thereby stimulating their infiltration of the cardiac vessel walls (Balistreri *et al.*, 2010). Nagase *et al.* (2006) also suggest that an imbalance in the levels of TIMP and MMPs can lead to excessive damage to the collagen tissues and therefore ballooning of the heart which may lead to a rupture of the heart.

The inflammation mediated by TNF alpha has been shown by previous studies to be due to the stimulation of chemotaxis of macrophages and neutrophils and the promotion of their phagocytic and cytotoxic influence. Also it is said to promote the collection of white blood cells by inducing increased expression of intracellular adhesion molecules (ICAMS) and endothelial leukocyte adhesion molecules (ELAMS) at sites of inflammation. The inflammatory condition is said to encourage the development of atheromatous plaques which may obstruct the coronary vessels and damage the heart (as a result of angina or myocardial infarction) (Libby and Theroux 2005).

TNF alpha also causes cell death and this is said to be due to various mechanisms some of which are the release of oxygen free radicals, nitric oxide, protein kinases, transcription of a variety of cytotoxic genes, regulation of nuclear regulatory factors and DNA fragmentation (Feldman *et al.* 2000).

1.4 Recent controversies

A lot of studies have been done on the effect of leptin and adiponectin in the development of diabetes and CVD. Adiponectin has been widely reported to reduce the risk of developing CVD and T2DM (Kim *et al.*, 2009, Nakasone *et al.*, 2013, Chen *et al.*, 2012 and Zhu *et al.*, 2008) but some of the studies done recently do not completely agree and this also forms a basis for carrying out the present study. It has been suggested in an *in-vivo* study that high levels of adiponectin correlated well and was very predictive of CVD. Also it has been shown that Simvastatin which is a medication that lowers plasma lipid levels, improved endothelium dependent vasodilatation and reduces levels of adiponectin and these differences have been explained by the variations in the characteristics of the populations which have been targeted (Koh et al 2010). Also some of the studies have shown that adiponectin levels are elevated in correspondence to the increase in inflammatory factors observed in chronic inflammatory states and states linked to auto-immunity such as type 1 diabetes, systemic lupus erythematosus, rheumatoid arthritis, and inflammatory bowel disease, this suggesting that adiponectin may be involved in the increases in chronic inflammation or at least does not influence or reduce it in any way in obesity. It has been suggested that adiponectin may confer protection in inflammation when adipose tissue is lost in starvation and fasting, but not in inflammation unassociated with obesity (Aprahamian and Sam 2011).

1.5 Research gap, questions, aims and objectives

1.5.1 Aims of the study

The study aimed to establish more precisely what effect various concentrations of adipokines (such as leptin and adiponectin) individually and in combination have on beta cell function and cardiovascular function whilst comparing these concentrations to concentrations found in lean and obese patients (individuals such as obese non diabetic, obese diabetic, non obese diabetic, non obese non diabetic (control)). This effect could be assessed by correlating the level of concentration of adipokines that have effect with markers of beta cell function such as HOMA-B and markers of CVD such as VEGF and VCAM and role of inflammatory factors. The effect of adipokines on gene expression for angiogenesis and atherosclerosis was also explored in the laboratory.

1. To investigate the effects of leptin and adiponectin individually and in combination in beta cell failure and cardiovascular diseases.

One of the goals of the present study was to find what the effect of leptin and adiponectin is on insulin secretion and gene expression in beta cells (to explore the basis of effect on insulin secretion) and in endothelial cells for genes for angiogenesis and atherosclerosis.

2. To examine the concentrations of leptin and adiponectin that has significant effects individually and in combination in beta cell failure and cardiovascular diseases.
3. Determining the concentrations of adiponectin and leptin in lean and obese patients.
4. To investigate the basis for the effect of the adipokines on insulin secretion and beta cell gene expression and on the expression of genes linked to atherosclerosis and angiogenesis.

1.5.2 Objectives of the study

In order to achieve the above aims the following were the objectives of this study.

Beta cells were grown and cultured into pseudo-islets which were then treated with various adipokines and insulin secretion assessed by ELISA.

Endothelial cells would be grown and RNA collected from them after treatment with adipokines. The RNA was to be purified with DNA-ses and reverse

transcribed and then PCR was carried out using the appropriate primers followed by gel electrophoresis to visualize gene expression.

Patients who met the obese and lean criteria for this study were to be recruited and blood samples taken to assess adipokine concentrations, beta cell failure markers and endothelial cell dysfunction markers.

The effects of the adipokines were to be explored further in the lab by exploring pathways that may be involved in these effects.

1.5.3 Research Gap

Most of the previous studies have looked into the effect of either leptin or adiponectin alone on endothelial cells and Beta cells (and not pseudo islets) (Balistreri *et al.*, 2010, Belkina and Denis 2010), and the concentrations at which these adipokines had their effects vary considerably between reports. In this study, we decided to investigate the effect of various concentrations of leptin and adiponectin alone and in combination (in pseudo islets which have been shown to have a better dose-related insulin secretion to glucose in comparison to mono layer beta cells) to elucidate the counter-regulatory effects they have on each other and also to study the effect they have on insulin

secretion from pseudoislets (which are clonal beta-cells grown in a configuration which mimics the islets of Langerhans) at a range of adipokine concentrations which are related to those found in physiological (lean) and pathological (obese) conditions in comparison to real values in a range of lean and obese patients.

1.5.4 Research Questions

The following are the research questions that arise from the gap in the previous studies;

- 1) What are the specific concentrations of adipokines (including leptin and adiponectin, individually and in combination) that significantly influence insulin secretion in beta cells?
- 2) What are the specific concentrations of adipokines (including leptin and adiponectin, individually and in combination) that significantly influence gene expression for genes that control angiogenesis and atherosclerosis in Eahy endothelial cells?
- 3) What is the basis of the effect of adipokines seen in insulin secretion in beta cells and gene expression for atherosclerosis and angiogenesis in Eahy endothelial cells?

4) Is there any correlation between the level of adipokines and the risk of developing diabetes and cardiovascular disease in patients?

1.5.5 Research Hypothesis

In order to carry out this research the following are the hypothesis that was developed from the research questions;

- 1) Leptin inhibits insulin secretion and causes upregulation of genes that cause angiogenesis and atherosclerosis.
- 2) Adiponectin causes an increase in insulin secretion and causes down-regulation of genes that regulate angiogenesis and atherosclerosis.
- 3) Adiponectin reduces the risk of CVD caused by leptin.
- 4) There is a high amylin secretion and a high insulin: amylin ratio in diabetics (or diabetic model)

Chapter 2

Materials and methods

2.1 Beta cell Preparation, treatment and result analysis

2.1.1-Materials

Materials such as equipments, reagents and kits used for the beta cells experiments can be found in Appendix 1.

2.1.1.1 Beta cell lines

The clonal mouse pancreatic β TC-6 cells were purchased from the American Type Culture Collection (ATCC) from LGC Promochem, UK. The MIN-6 cells were received as a gift from Dr Catherine Arden of the University of Newcastle, England, United Kingdom.

2.1.1.2 Buffer for beta cell treatment

The buffer used for the experiments on insulin secretion was the Krebs-Hepes buffer (KHB) which was made up of sodium chloride (NaCl- 119mM per 100ml), potassium chloride (KCl- 4.74mM, 237µl of a 2M solution per 100ml), calcium chloride (CaCl₂- 2.54mM 254µl of 1M solution per 100ml), magnesium chloride (MgCl₂ 1.19mM- 60µl of a 2M solution per 100ml), monobasic potassium phosphate (KH₂PO₄ -1.19mM - 120 µl of 1M solution per 100ml), sodium hydrogen carbonate (NaHCO₃ 25mM- 2.5ml of a 1M solution per 100ml), Hepes (10mM- 1ml of a 1M solution per 100ml) bovine serum albumin (BSA- 0.5% - 1ml of 1M solution per 100ml). The salts were dissolved in distilled water and BSA added last in order to prevent it from binding to any undissolved salt, thereby reducing its concentration. This mixture was then filtered and PH corrected to 7.4.

2.1.2 Cell culture/ Pseudo-islet Preparation

The cell culture process involved the following processes; recovery of cells from liquid nitrogen, maintaining the cells (change of media, sub-culturing cells and freezing cells back into liquid nitrogen for use in experiments at a later date) and preparation of pseudo-islets.

2.1.2.1 Beta cell recovery from liquid nitrogen.

The Beta cells (MIN6 cells passages 23 and 24) were gifts from Dr. Catherine Arden of the Newcastle University. The cells frozen in vials were thawed out quickly and resuspended with 5-10ml of cell culture medium (see components in section 2.1.2.2) in a universal tube and this was centrifuged at 500g for 5 minutes to make the cells into a pellet and separate them from the freezing solution. The supernatant which contains the media and the freezing solution was then removed and the pellet resuspended in 5ml of media and this was mixed thoroughly in the universal tube to ensure an even distribution of cells and then placed in a 25cm² flask. The cells were then checked under the microscope and placed in the incubator with 5% CO₂ and 95% air at 37°C.

2.1.2.2 Maintenance of the Beta Cells

The MIN6 cells were cultured in DMEM (supplemented with 5ml L-glutamine and Penicillin/Streptomycin, 75ml of fetal bovine serum and 2μL of dimercaptoethanol). The βTC cells used were however cultured in RPMI 1640 medium supplemented with 10% foetal bovine serum, 100μg/ml Penicillin with 100μg/ml streptomycin, 11mM glucose, 2mM L-glutamine.

Media was changed when necessary by removing the media with a sterile pipette and replacing with fresh media (5ml for 25cm² flask or 10ml for 75cm² flask).

When the cells had become 80% confluent in the 25cm² flask they were subcultured into three 75cm² flasks. This involved removing the media from the 25cm² flasks and then washing the excess protein from the cells 3 times with 2.5ml of Hanks Buffered Salt Solution (HBSS) and this was followed by the addition of 0.4ml of 2.5g/L trypsin to the cells and the cells were then placed in the incubator for about 3-5 minutes checking regularly to make sure the cells had detached.

Once detached, about 5ml of media was added to dilute the trypsin and this mixture transferred into a universal tube and spun in a centrifuge for 5 minutes at 500g to separate the pellet from the media/trypsin mixture. The pellet was then re-suspended in 10ml of media and thoroughly mixed and 3.33ml of cell-media in each 75cm² flask. This was then topped up with 6.67mls of media to make it 10mls in each 75cm² flask. The flasks were then placed back in the incubator and 10mls of media changed whenever necessary until the cells in the 75cm² flasks were at least 80% confluent and ready to be used for experiments or to be frozen down if they were not be used for experiments.

The process of freezing cells down involved removing the medium from the 75cm² flasks and then washing the excess protein from the cells 3 times with 5ml of Hanks buffered salt solution (HBSS). This was followed by the addition of 0.8ml of 2.5g/L trypsin solution to the cells and the cells were then placed in the incubator for about 3-5 minutes checking regularly to make sure the cells had detached. Once detached, about 5ml of complete medium was added to dilute the trypsin and this mixture transferred into a universal tube and spun in a centrifuge for 5minutes at 500g to separate the pellet from the media/trypsin mixture.

The freezing-down involved resuspending the cells in the pellet in 1ml of 100% Fetal Bovine Serum (FBS) and adding 1ml of 20% Dimethyl Sulphuroxide(DMSO). This mixture was then split into two cryogenic vials (1ml each) and the vials placed in the -20°C freezer overnight and transferred into the -80°C freezer and then into the Liquid nitrogen tank(-180°C) on the 3rd day.

For the experiment about 3×10^6 cells were seeded per 75 cm² vented flask and grown in a humidified incubator at 37° centigrade in 5% CO₂ and 95% atmospheric air and were routinely passaged when they attained approximately 70-80% confluence when viewed under the microscope.

2.1.2.3 Pseudo-islet preparation

This involved preparing 2% bovine gelatin and autoclaving overnight. The pseudo-islets were prepared by coating 24 well plates with 500uL of 2% gelatin and incubated overnight. BTC Cells were then seeded in culture medium into the wells after the excess gelatin was removed. The 24 well plates were then returned to the incubators and allowed to grow into Pseudo-islets. Medium was changed on the 3rd and 5th days and after 5-9 days pseudo-islets were fully formed.

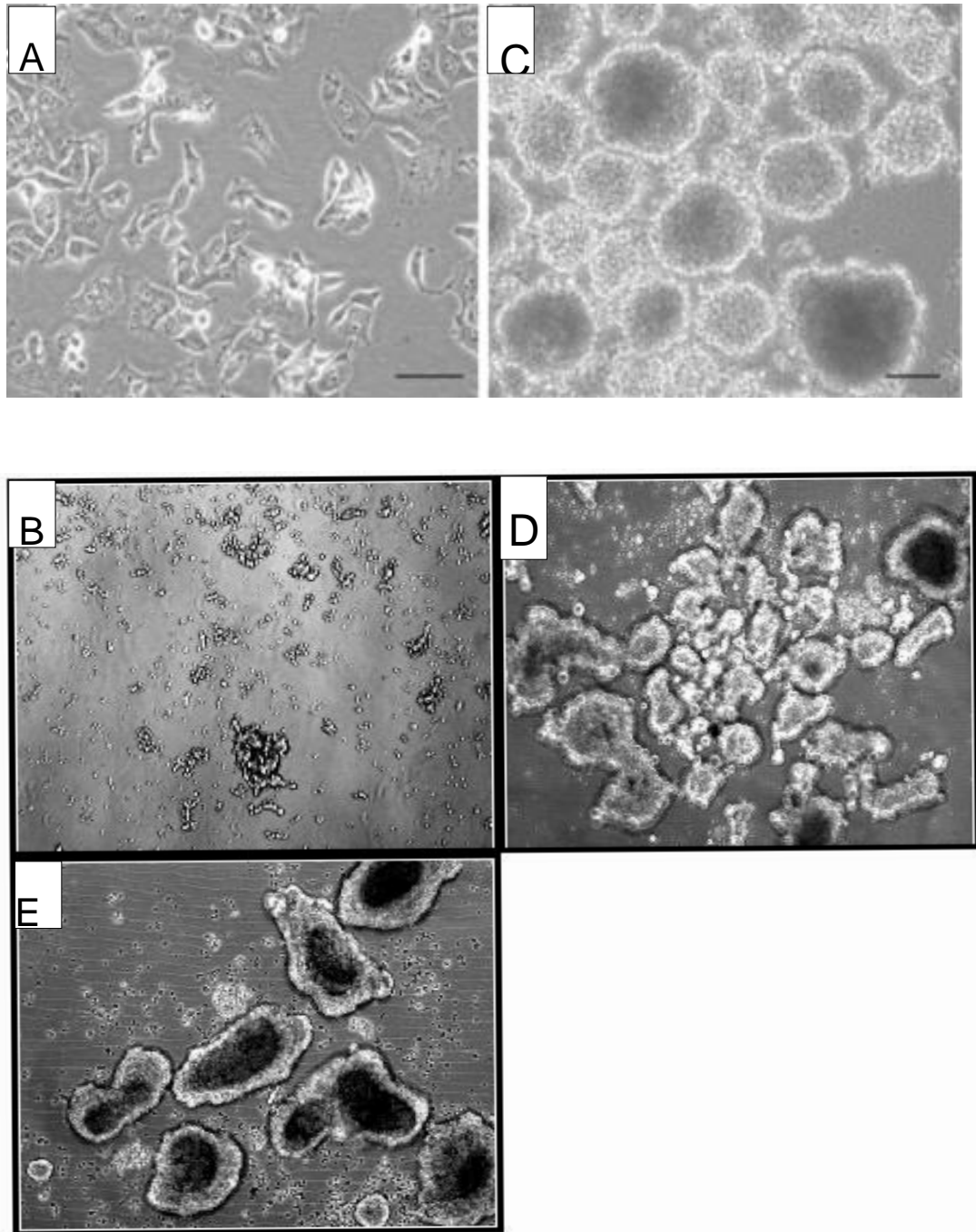


Figure2.1. Stages of formation of pseudo-islets. The figure shows day 1(Fig A), Day 2 (Fig B), Day 5 (Fig C), Day 7(Fig D), Day 9(Fig E).

2.1.3 Treatment of cells with adipokines

The Pseudo-islets were incubated initially with low glucose (2.2mM) in KHB (Krebs Hepes Buffer) for a minimum of 2 hours to synchronize the insulin secretion to basal levels and then incubated with 5 different concentrations of Adiponectin (0.1, 0.2, 0.5, 1 and 5µg/ml) leptin (10, 50, 100, 200, 500ng/ml) in triplicates in low (2.2mM) or high glucose (22mM) all in KHB for 1 hour (see Figure 2.2). 250µL of KHB containing secreted insulin was then collected and centrifuged at 500g for 5 min to remove any loose cells left and then 200uL of the supernatant was removed and stored frozen at -20° C until the samples were assayed for insulin by immunoassay (mouse insulin ELISA, Mercodia, Uppsala, Sweden).

LG	LG	LG	HG	HG	HG	
0	0	0	0	0	0	CONTROL
10	10	10	10	10	10	LEPTIN
50	50	50	50	50	50	L
100	100	100	100	100	100	L
200	200	200	200	200	200	L
500	500	500	500	500	500	L
0.1	0.1	0.1	0.1	0.1	0.1	ADIPONECTIN
0.2	0.2	0.2	0.2	0.	0.2	A
0.5	0.5	0.5	0.5	0.5	0.5	A
1.0	1.0	1.0	1.0	1.0	1.0	A
5.0	5.0	5.0	5.0	5.0	5.0	A

Figure 2.2 Scheme for treating beta cells. Figure shows the treatment with adipokines leptin and adiponectin. The treatment was done in triplicate for 5 different concentrations of each adipokine. Leptin (10, 50, 100, 200, 500 ng/ml), Adiponectin (0.1, 0.2, 0.5, 1.0, 5.0 µg/ml).

2.1.4 Insulin assay

The assay was performed with Mercodia mouse insulin ELISA kit (From Upsalla, Sweden). The assay was carried out according to the recommendations of the manufacturer as stated in the protocol contained in the kit.

2.1.5 Amylin assay

The amylin assay was performed using the amylin enzyme immunoassay kit (Phoenix Pharmaceuticals USA). The assay was carried out according to the recommendations of the manufacturer as stated in the protocol contained in the kit.

2.1.6 Result analysis

This was done using the Mercodia Excel calculator (for insulin assay) and the Phoenix Pharmaceuticals' Excel calculator (for amylin assay), which involved imputing the calibrators and using this to plot the mean absorbance values against the insulin concentration of the calibrators by drawing an inverted curve automatically on a log-log sheet. This calibrator curve converts the spectrophotometer reading for all the samples to insulin values automatically when inputted. These values were now multiplied by 10 and expressed in $\mu\text{g/L}$ and further analyzed by finding the average of the triplicates and drawing a graph to represent the various levels on insulin secretion by each sample. The minitab 16 software (ANOVA) and the Student's t-test were then used to find the statistical significance ($p < 0.05$) and correlations between the effect of glucose and adipokine treatments (The methods used were specified individually in the legends of the graphs for various experiments).

2.2 Endothelial cell preparation, treatment and result analysis

2.2.1-Materials

Materials such as equipments, reagents and kits used for the beta cells experiments can be found in Appendix 1.

2.2.1.1 EAHY cell lines

EAHY cells (endothelial cells) were a gift from Dr. James Brown from Aston University.

2.2.1.2 Antibodies

The AMPK inhibitor used was 10µg/ml Dorsomorphine Dihydrochloride from Tocris Bioscience Bristol, UK. The ERK inhibitor was ERK Inhibitor II, FR180204 and the p38 MAPK inhibitor was TAK 715 both from Santacruz Biomedicals, USA.

2.2.2 EAHY Cell Culture

The cell culture process involved the following processes; recovery of cells from liquid nitrogen, maintaining the cells (change of media, sub-culturing cells and freezing cells back into liquid nitrogen for use in experiments at a later date).

2.2.2.1 EAHY Cell recovery from liquid nitrogen.

EAHY endothelial cells were frozen in vials and were thawed out quickly and resuspended with 5-10ml of culture medium (see contents in 2.2.2.2) in a universal tube and this was centrifuged at 500g for 5 minutes to make the cells into a pellet and separate them from the freezing solution. The supernatant which is made up of a mixture of the media and the freezing solution was then removed and the pellet resuspended in 5ml of media and this was mixed thoroughly in the universal tube to ensure an even distribution of cells and then placed in a 25cm² flask. The cells were then checked under the microscope and placed in the incubator with 5% CO₂ and 95% air at 37°C.

2.2.2.2 Maintaining the EAHY Cells

The endothelial cells (EAHY) used were cultured in RPMI 1640 medium supplemented with 10% foetal bovine serum, 100U/ml penicillin with 100µg/ml streptomycin, 2mM L-glutamine.

Complete culture medium was changed when necessary by removing the media with sterile pipette and replacing with fresh culture medium (10ml for 25cm² flask or 20ml for 75cm² flasks). When the cells became about 80-100% confluent in the 25cm² flask they were subcultured into three 75cm² flasks. This involved removing the media from the 25cm² flasks and then washing the excess protein from the cells 3 times with 2.5ml of Hanks Buffered Salt Solution (HBSS) and this was followed by the addition of 0.5ml of 2.5g/L trypsin solution to the cells and the cells were then placed in the incubator for about 3-5 minutes checking regularly to make sure the cells had detached.

Once detached, about 5mls of media was added to dilute the trypsin and this mixture transferred into a universal tube and spun in a centrifuge for 5 minutes at 500g to separate the pellet from the culture medium/trypsin mixture. The pellet was then re-suspended in 10ml of culture medium and thoroughly mixed and 3.33ml of cell/culture media in each 75cm² flask. This was then topped up with 16.67ml of the culture medium to make it 20ml in each 75cm² flask. The

flasks were then placed back in the incubator and 20ml of culture medium changed whenever necessary until the 75cm² flasks were at least 80-100% confluent and ready to be used for experiments or to be frozen down if they were not going to be used for experiments.

The process of freezing the EAHY cells down involved removing the culture medium from the 75cm² flasks. The excess protein was then washed from the cells 3 times with 5ml of Hanks Buffered Salt Solution (HBSS). This was followed by the addition of 2.5ml of 2.5g/L of trypsin solution to the cells and the cells were then placed in the incubator for about 3-5 minutes checking regularly to make sure the cells had detached. Once detached, about 5mls of culture medium was added to dilute the trypsin and this mixture transferred into a universal tube and spun in a centrifuge for 5 minutes at 500g to separate the pellet from the culture medium/trypsin mixture.

The freezing down involves resuspending the cells in the pellet in 1ml of 100% Fetal Bovine Serum (FBS) and adding 1ml of 20% Dimethyl Sulphoxide (DMSO). This mixture was then split into two cryogenic vials (1ml each) and the vials placed in the -20°C freezer overnight and transferred into the -80°C and then into the liquid nitrogen tank (-180° C) on the 3rd day.

For the experiment, about 3×10^6 cells were seeded per 75cm^2 vented flask and grown in RPMI culture medium (supplemented with 5ml L-glutamine and Penicillin/Streptomycin, 50ml of Fetal bovine serum) in a 75cm^2 flask, and were seeded into 6 well plates when they were 80-100% confluent.

2.2.3 Treatment of endothelial cells with adipokines

The EAHY cells were cultured in RPMI culture medium (supplemented with 5ml L-glutamine and Penicillin/Streptomycin, 50ml of foetal bovine serum) in a 75cm^2 flask, and about 3×10^6 cells were seeded from each 75cm^2 into 6 well plates (when they were 80-100% confluent). When the cells had grown for about 3 days in the 6 well plates, they were then incubated in low serum (2%) RPMI culture medium for about 6 hours to starve the cells of protein and bring all the cells to the G0 phase of the cell division process before the experiment was started. The EAHY cells were then treated with low and high concentrations of leptin (0.5nM and 1nM) and/or low and high concentrations of adiponectin (1 and 100nM) (see figure 2.3) with wells having treatments with individual adipokines and also adipokines in combination for 24 hours and the media collected and frozen down for analysis and the cells collected and centrifuged into pellets at 500g for 5 minutes. The cell pellets were then re-suspended and washed in sterile phosphate-buffered saline and centrifuged at 500g for 5 minutes.

EXPERIMENT SCHEME1

LL	HL	LA	HA	LL+LA	LL+HA	HL+LA	HL+HA
C	C	C	C	C	C	C	C

EXPERIMENT SCHEME2

LA	HL	LA+HL
CLA	CHL	CLA+HL

EXPERIMENT SCHEME 3

HA	LL	HA+LL
CHA	CLL	CHA+LL

EXPERIMENT SCHEME 4

LA	HL	LA+HL
LA + INHIBITOR	HL+ INHIBITOR	LA+HL+ INHIBITOR

Figure 2.3 Scheme for the treatment of EAHY with Adipokines. Figure shows treatment with various concentrations of leptin and adiponectin (LL- low leptin (0.5nM), HL- high leptin (1nM), LA-low adiponectin (1nM), HA- high adiponectin (100nM), combinations-LA+HL, HA+LL.

2.2.4 Gene Expression Quantification

There were 6 phases; mRNA extraction, removal of DNA present in the RNA sample (RNA purification), reverse transcription, PCR, gel electrophoresis and Band analysis.

2.2.4.1 Total RNA extraction and purification and quantification

Total RNA was extracted from the pellet of treated cells using the Norgen Kit (Norgen UK, Fradley, Staffordshire) and quantified. The pellet of cells were counted with a Coulter counter to make sure that the cells used were not more than the 3 million stipulated to be used with the Norgen kit and then washed with PBS and centrifuged at 500g for 5 minutes. The PBS was then removed completely. 350µl of lysis solution was then added to the pellet and left to stand for 5 minutes at room temperature to lyse the cells. 200 µl of 95% ethanol was then added to the lysine and this mixed thoroughly with a vortex for about 10 seconds. This mixture was then transferred into an assembly of column and collection tubes and this assembly spun at 14000g for 1 minute. The column was then washed with 400 µl of wash buffer at 14000g for 1minute 3 times and spun an additional 2 minutes to dry. The collection tube was then replaced with a 1.7ml elution tube and 50ul of elution solution added to the column-elution

tube assembly and spun at 200g for 2minutes and 14000g for 1 minute and then 14000g for another 1 minute.

The RNA was quantified using a Thermoscientific 2000c/2000 nanodrop spectrophotometer. This involved cleaning the spectrophotometer with distilled water on a tissue, and then blanking the spectrophotometer by placing 1.5 µl of elution fluid on the nanodrop window and placing 1.5 µl of samples to be measured on the nanodrop window and measurements were made with the nanodrop.

The RNA extracted was placed in a -80° C freezer until purification

2.2.4.2 RNA Purification

The purification of the mRNA was done with an RNase- free DNase. An RQ RNase-Free DNase (Promega Southampton, UK) was used to remove any DNA from the RNA sample.

The process involves placing all the enzymes (RQ1 RNase-free DNase 10x reaction buffer and RQ1 RNase-free DNase). Heating blocks were preset at 37 and 65 degrees.

The enzymes were used at 1ul each per 1-8ul of RNA samples (5 µl was used to minimize the effect of magnesium on the effect of the DNase. Samples were placed on ice and 5ul of DNase was added to each sample and then 5ul of DNase buffer added to each sample and placed immediately back into the ice to prevent any premature reaction. The samples were then placed in the 37 degrees block to incubate for 30 minutes and then 5ul of the stop solution added to the samples to inactivate the DNase and samples placed in the 65 degrees for 10 minutes.

2.2.4.3 RNA Reverse Transcription

This process was done in 2 phases; the annealing and the extension phases.

The annealing phase is the phase that involves heating up RNA and then cooling suddenly to allow the primers of the DNA of interest to attach to the ends of the RNA sequence and to allow the DNA sequence of interest to be formed in the extension phase. The process involves first calculating how much

of the RNA solution will contain 1000ng (1µg)/ µl of RNA and this was done by dividing 1000 by the concentration of the RNA in the sample (for example if 1µl of the sample contains 200ng of RNA, therefore 1ng will be in 1/200ul and 1000ng will be in 1/200x1000=5ul of the RNA sample). Once the volume of the RNA sample that contained 1000ng of RNA was determined, 1µl was added to this number and this total was taken away from 10µl (total for annealing phase) to find out how much of RNA-se/DNA-se free water was needed to be added to the mixture to make a total of 10µl (Annealing phase requires the addition of 1µl of primer + Volume of RNA that contains 100ng + RNase/DNase free water. =10µl).

COMPONENT	1 REACTION
RNA template (3pg -2ng)	X µl (Volume of RNA sample that contains 1000ng)
RT primer	1 µl
RNase/DNase free water	Y µl (10-(RNA+ 1)
Final volume	10 µl

Table2.1 Table showing the components of the mixture for the annealing phase of the reverse transcription.

The annealing mixture is placed in a block of 65°C for 5 minutes and transferred straight into Ice to ensure the primers bind properly to the RNA.

The extension phase involves preparing a master mix of the following;

COMPONENT	1 REACTION
NanoScript 10X Buffer	2.0 µl
dNTP mix 10mM of each	1.0 µl
DTT 100mM	2.0 µl
RNAse/DNAse-free water	4.0 µl
NanoScript enzyme	1.0 µl
Final volume	10.0 µl

Table2.2 Table showing the contents of the master mix for the extension phase of the reverse transcription. The number of reactions (plus an additional reaction for pippetting error) was multiplied by each content and total added to make the master mix. From the mastermix 10.0 µl was taken and added to the 10.0 µl for each sample from the annealing phase to make 20.0 µl for the extension phase cycling.

This mixture is made up to 10 µl for each sample and each component is multiplied by the total number of samples we have and from this master mix, 10

µl is added to each 10 µl sample on ice from the annealing phase of the reverse transcription and this mixture vortexed and placed in a thermocycler. The thermocycler is programmed to run at 55 degrees for 20 minutes and then 75 degrees for 15 minutes to extend and grow the DNA.

The DNA generated was then quantified using the Nanodrop spectrophotometer and stored in the -20°C freezer for PCR.

2.2.4.4 Polymerase Chain Reaction (PCR)

The PCR process is a process of amplifying a gene of interest. The experiment aims to treat the cells with adipokines in order to see what influence the adipokines will have on the expression of angiogenic and atherosclerotic genes

Gene	Forward Primer	Reverse Primer
Angiogenic Genes		
HIF-1	GGCGCGAACGACAAGAAA AAG	CCTTATCAAGAT GCGAACTCACA
MMP2 *	CCGTCGCCCATCATCAAGT T	CTGTCTGGGGCA GTCCAAAG
MMP9	TGGCAGAGATGCGTGGAG A	GGCAAGTCTTCC GAGTAGTTTT
TIMP1	CTTCTGCAATTCCGACCTC GT	CCCTAAGGCTTG GAACCCTTT
TIMP2*	AAGCGGTCAGTGAGAAGG AAG	GGGGCCGTGTA GATAAACTCTAT
TIMP3*	TGCAACTTCGTGGAGAGG TG	CACAAAGCAAGG CAGGTAGTA
TNFa	ATGAGCACTGAAAGCATGA TCC	GAGGGCTGATTA GAGAGAGGTC

VEGF-A	CAACATCACCATGCAGATT ATGC	GCTTTCGTTTTTG CCCCTTTC
VEGF-C	AGGCTGGCAACATAACAG AGA	TCCCCACATCTA TACACACCTC
Atheroslerotic Genes		
ICAM-1	TTGAACCCACAGTCACCT AT	CCTCTGGCTTCG TCAGAATCA
VCAM-1	GCTGCTCAGATTGGAGACT CA	CGCTCAGAGGGC TGTCTATC
E-Selectin	ACCTCCACGGAAGCTATGA CT	CAGACCCACACA TTGTTGACTT
LPL *	ACAAGAGAGAACCAGACT CCAA	AGGGTAGTTAAA CTCCTCCTCC
LPA*	ATGGAATCAACTCTCCTCA CAAC	ATCACCTCGGTA GCAGTCCT
Cadherin 5	GACCGGGAGAATATCTCA GAGT	CATTGAACAACC GATGCGTGA

GAPDH	GAGTCAACGGATTTGGTC G	GGAAGATGGTGA TGGGATTT
BACTIN	AATCTGGCACCACACCTTC TCC	CTCCTTAATGTCA CGCACGAT

Table 2.3 Table showing the various genes of interest that influence atherosclerosis and angiogenesis. The genes marked (*) are the genes that were well expressed. GAPDH and β -ACTIN were used as house-keeping genes.

These were the genes of interest. The PCR involved exploring the expression of each of these genes initially in each of the 16 samples (see experiment scheme 1) and later reduced to 5 genes (2 atherosclerotic and 3 angiogenic genes) and 6 samples (As seen in experiment scheme 2) as there were only 5 of these genes that were properly expressed. As a form of control for the PCR, PCR was done for all the samples to measure the expression of house-keeping genes, GAPDH and β -Actin.

The PCR involves preparing a master mix which is added to the sample and mixture placed in a thermocycler. The master mix was made up of the following;

Component	Volume(μ l)/Sample
Taq Polymerase	25ul
Magnesium Chloride	0.5
Forward Primer	1
Reverse Primer	1
dNTP	1
RNase-DNase Free water	19.5
Final Volume/	48 μ l

Table 2.4 Tables showing the mastermix for the reverse transcription PCR.

The number of samples (with one added for pipetting error) was multiplied by each component to make a master mix. Out of the mastermix, 48 μ l was added to a tube and 2 μ l of each cDNA was added to each tube and this mixed thoroughly and then placed in the thermocycler.

These were all placed in ice initially and after the total master mix was prepared, 48ul was added to each tube and 2ul (50ng) of DNA from each

sample was added and placed in ice to prevent premature reaction and the mixture was then vortexed and placed in a thermocycler which was set to the

Following parameters;

Setting	Status	Duration	Temperature
Lid: (Time depends on the size of the gene)			105.0 degrees
Heated lid before program:	On		
Pause before program	Off		
Initial denaturation		5minutes	95 degrees
Hot start	Disable		
Number of cycle		32	
Denaturation		30seconds	95 degrees
Annealing:		30 seconds	55 degrees
Extension	(extension 1min/kb)	1minute	72 degrees
Final extension		5minutes	72 degrees.

Table 2.5 Table showing the settings for the thermocycler for PCR

The PCR product which was generated was then placed in the -20 degrees freezer ready for gel electrophoresis.

2.2.4.5 Gel electrophoresis

Gel electrophoresis was performed to visualize the amplified products of PCR and to quantify them. This involves preparing an agarose gel and placing the PCR product in the wells and then running the electrophoresis in TAE (Tris-Acetate EDTA) buffer.

The gel preparation involved preparing a 1% agarose with TAE buffer. The TAE buffer was used as the DNA molecules bind to the salt in the mixture in the TAE buffer and moves with the electric charge from the negative pole to the positive through the gel. The 1% gel was prepared with 7 μ l of Ethidium Bromide in order to reveal the bands under the UV light. The gel was allowed to cool just enough not to solidify and then poured into a mould with a comb in it to form the wells where the samples was placed in a Life Technologies electrophoretic tank. The gel was allowed to cool and solidify for about 30 minutes. The mould with the gel was then placed in the electrophoresis tank and TAE buffer added to just below the maximum mark and the comb removed to expose the wells. The electrophoresis tank was then covered and the leads connected accordingly from the tank to the Voltameter (black to black and red to red). The Voltameter was set at 100V and run for 1hour. The gel was taken on a tray to the UV illuminator after electrophoresis for 1hour. Pictures of gel were taken and saved for the analysis of the bands.

2.2.4.6 DNA band analysis

The band (representing the relative expression of genes of interest) on the images taken with the UV camera was analyzed with the Image J software which measures and records the pixel density of each band.

The Relative expression of gene of interest was also compared to the housekeeping gene GAPDH or β -actin, thus calculating the ratio number (= pixel density for Sample/Pixel density for GAPDH or β -actin). The average of three different experiments was recorded as the ratio number. The ratio number was then plotted on a graph in order to compare the gene expression in controls to the various treatments made.

2.2.4.7 Real-time PCR

2.2.4.7.1 Primers

All primers were designed with the help of the reference sequence from the NCBI nucleotide sequence base. Taqman primers with high specificity were used in these experiments.

Primer	Strand	Primer Sequence
Mouse Actin	Sense	AATCTGGCACCCACACCTTCTCC
	Anti-sense	CTCCTTAATGTCACGCACGAT
MMP2	Sense	CCGTCGCCCATCATCAAGTT
	Anti-sense	CTGTCTGGGGCAGTCCAAAG
TIMP2	Sense	AAGCGGTCAGTGAGAAGGAAG
	Anti-sense	GGGGCCGTGTAGATAAACTCTAT
TIMP3	Sense	TGCAACTTCGTGGAGAGGTG
	Anti-sense	CACAAAGCAAGGCAGGTAGTA
LPA	Sense	AGGGTAGTTAACTCCTCCTCC
	Anti-sense	ATCACCTCGGTAGCAGTCCT
LPL	Sense	ACAAGAGAGAACCAGACTCCAA
	Anti-sense	ATGGAATCAACTCTCCTCACAAC

Table 2.6 Primers used for the real time PCR. Primers were purchased from Qiagen life technologies UK.

2.2.4.7.2 mRNA extraction, reverse transcription

The mRNA from the endothelial cells EAHY, purification and reverse transcription to cDNA was done in the same way as was described for the reverse transcription PCR above.

2.2.4.7.3 Real time PCR studies for gene expression analysis for angiogenic and atherosclerotic genes

The real-time RT-PCR reactions were performed using the iCycler Real-Time PCR Detection System. Reaction volumes used were 25µl containing 2µl of cDNA 1.25µl of each pair of primers, 9.5µl nuclease free water and 12.5µl of Mastermix. Two housekeeping genes (β -actin and GAPDH) were amplified in separate reaction to normalize results.

Thermal cycling conditions were as follows;

95° C for 1 minute (initial denaturing)

95°C for 30 seconds

58°C for 30 seconds

95°C for 30 seconds

72°C for 10 minutes (final extension)

Each sample reaction was run in triplicate and expression quantified as the number of cycles (C_T) after which fluorescence exceeds the background threshold minus the C_T for the housekeeping control (ΔC_T). The RT-PCR statistical analysis was performed using student T test for two group comparisons and analysis of variance (ANOVA) for multiple comparisons. The relative expression levels were calculated by the formula $2^{-\Delta\Delta C_T}$, comparing treatment samples and untreated control samples.

2.3 Clinical blood sampling for adipokine assay

2.3.1 Materials

Materials such as equipments, reagents and kits used for the beta cells experiments can be found in Appendix 1.

2.3.2 Study design and result analysis

This aspect of the study was done in collaboration with Drs Ananth Nayak and Baldev Singh (Diabetes Centre, New Cross Hospital, Wolverhampton) in a study in 2013 on glycation gap and mortality in diabetes. This therefore influenced many of the decisions and planning for the methods and result analysis in this section of the study.

2.3.2.1 Ethical committee approval

The use of the clinical database for this study was approved by the relevant local U.K. National Health Service Research Ethical Committee.

2.3.2.2 Selection of patients and data collection

Following ethical approval, it was important to select appropriate patients for the study. HbA_{1c} and fructosamine estimations had been undertaken at New Cross Hospital Wolverhampton by Nayak *et al.* over 4 years (2006–2009). In this period, adults with diabetes (≥ 18 years of age) who had paired estimations of HbA_{1c} and fructosamine performed on the same day from the same sample set were recruited for the study (Nayak *et al.*, 2013). Of these patients, 150 were recruited for this present study.

Information about the clinical condition of the patients was obtained from the diabetes registry and linked to the information derived from the analysis the patients' blood samples. The diabetes register is validated to be >99% accurate for the identification of known diabetes and for mortality status in linkage with the National Health Service Strategic Tracing Service (Nayak *et al.*, 2013). Exclusion criteria for this study, as in the study by Nayak *et al.* (2013) were: pregnant women, those with creatinine >200 $\mu\text{mol/L}$, those with known hemoglobinopathy, or those with an abnormal electrophoretic pattern on HbA_{1c} testing.

The 150 patients that were selected were patients who had 2 pairs of simultaneous estimations of HbA_{1c} and 2 calculated G-gaps that were consistent in direction (positive or negative direction). The 150 patients selected were made of the first 75 had 2 pairs of simultaneous estimations of HbA_{1c} and

2 calculated G-gaps that were consistently positive and the first 75 patients that had 2 pairs of simultaneous estimations of HbA1c and 2 calculated G-gaps that were consistently negative and not from random selections.

2.3.2.3 Ranking of patients' risk of developing complications such as retinopathy, renal complications and vascular complications

Concerning retinal complications, the English National Screening Program for Diabetic Retinopathy (ENSPDR) was used as a guide for the digital retinal screen. Retinopathy was classified as either having or not having retinopathies. For renal complications, the renal status was classified by considering the Urine albumin-creatinine ratio (UACR) and the status was classed as low risk or high risk for progressive microalbuminuria (<10 or >10 mg/mmol). For the cardiovascular complications, the classification was based on the presence or absence of any previous cardiac, cerebral, or peripheral macrovascular event.

2.3.2.4 Methods of analysis

The Diabetes Control and Complications Trial (DCCT) aligned HbA_{1c} was used in the analysis as the HbA_{1c} International Federation of Clinical Chemistry and Laboratory Medicine (IFCC) values were available only from 1 June 2009. HbA_{1c} was measured using high-performance liquid chromatography. According to the agreed conditions of collaboration with Nayak *et al.*, the performance scores in the UK National External Quality Assurance Scheme (UK NEQAS) were used and are as follows: A (accuracy) score <100 and B (bias) score <2%, which were within the acceptable limits of the UK NEQAS for glycated hemoglobins (maximum limits: A score <200 and B score less than $\pm 7.5\%$). The between-batch coefficient of variation was 1.8 and 1.4% for an HbA_{1c} of 5.7% (39 mmol/mol) and 9.5% (80 mmol/mol), respectively. Fructosamine was measured by nitrotetrazolium-blue reduction. A Cobas kit with between-batch coefficient of variation 3.1% at a level of 263 $\mu\text{mol/L}$ and 2.2% at 518 $\mu\text{mol/L}$ was used (Nayak *et al.*, 2013).

2.3.2.5 Fructosamine-predicted HbA_{1c} and the G-gap calculations

According to Nayak *et al.* (2013), the predicted HbA_{1c} (FHbA_{1c}) was calculated from the simultaneously measured fructosamine standardized to the HbA_{1c}

distribution according to the following equation: $FHbA_{1c} = \{[(\text{fructosamine} - \text{mean fructosamine}) / \text{SD fructosamine}] \times \text{SD HbA}_{1c}\} + \text{mean HbA}_{1c}$. In order to then calculate the G-gap, the predicted HbA_{1c} ($FHbA_{1c}$) was then subtracted the true HbA_{1c} ($G\text{-gap} = HbA_{1c} - FHbA_{1c}$). Importantly the $FHbA_{1c}$ was not derived from HbA_{1c} by correlation/regression methods. A negative G-gap is said to represent the true HbA_{1c} reading lower than the $FHbA_{1c}$, and a positive G-gap denotes the true HbA_{1c} reading higher than that predicted by fructosamine. Among those with a second paired HbA_{1c} -fructosamine estimation, in order to identify those with a consistent G-gap direction, the product of two G-gaps was calculated. If consistent, the G-gap product would be positive (positive \times positive = positive; negative \times negative = positive), but any discordance in direction of the G-gap over time in two paired readings would yield a negative G-gap product (negative \times positive = negative) (Nayak *et al.*, 2013).

2.3.2.6 Categorization of the G-gap and statistical analysis

To simplify things, the G-gap (unit = HbA_{1c} %) was categorized as negative when less than or equal to -1 (i.e., more negative than -1), neutral greater than -1 to less than $+1$, or positive when greater than or equal to $+1$.

In order to analyse the data statistically, the correlation between the leptin/adiponectin ratio and the parameters relating to the risk of developing retinal, renal and cardiovascular complications were done using the minitab

version 17 ANOVA and graphs drawn to represent these correlations, All statistical tests were considered significant at $P < 0.05$.

2.3.3 Blood Sampling

After recruitment, patients were given dates when blood sampling was done via the safe vacutainer system of venepuncture. These blood samples were then stored initially in a -20 degrees freezer and then transferred to a -80 degrees freezer for longer storage.

2.3.4 Plasma sample analysis for Adipokines leptin and adiponectin

The plasma sample was prepared by collecting plasma using heparin as an anticoagulant and centrifuging for 15 minutes at 1000 x g within 30 minutes of collection. The plasma samples were then stored frozen at -20 degrees after aliquoting (to avoid repeated freeze-thaw cycles) and were then assayed for the levels of leptin and adiponectin using the R and D Pharmaceuticals (Abingdon, UK) enzyme immuno-assay ELISA kit. The assay was carried out according to the recommendations of the manufacturer as stated in the protocol contained in the kit.

Chapter 3

Regulation of gene expression for angiogenesis and atherosclerosis by leptin and adiponectin in endothelial cells

3.1 Introduction

Cardiovascular disease (CVD) affects the blood vessels and the heart and atherosclerosis and angiogenesis (both stimulated by inflammation) have both been strongly linked with the pathogenesis of CVD. Atherosclerosis results from the development of an inflammatory point in the vascular lining (for example an injury from high blood pressure) which stimulates smooth muscle cell activation, migration and proliferation which may lead to occlusion of the vessels and CVD (Li *et al.*, 2005). Angiogenesis is also said to be critical for the neovascularisation of an atheromatous plaque further predisposing to CVD (Park *et al.*, 2001). This therefore makes it important to look more closely at the possible role of leptin and adiponectin in the expression of genes that code for proteins that influence atherosclerosis and angiogenesis.

Leptin and adiponectin are adipokines secreted by fat cells from visceral adipose tissue and previous studies suggest leptin to be pro-inflammatory and to encourage the expression of angiogenic and atherosclerotic genes (Dubey and Hesong 2006).

Adiponectin however, has been reported to reduce inflammation and risk of cardiovascular disease as a result of its cardio-protective qualities (Adya *et al.*, 2012, Zhu *et al.*, 2008, Okamoto *et al.*, 2006). Other studies have suggested

that adiponectin, though not pro-inflammatory, may not confer a cardioprotective quality (Antoniades *et al.*, 2009).

Due to the controversies around the roles of these adipokines, attempts have been made to investigate the effect of various concentrations of these adipokines and the basis of these effects. Studies have shown that leptin, through the JAK-STAT pathways via MAPK encourages the entry of dimerised STAT3 into the nucleus to influence the expression of angiogenic and atherosclerotic genes, while adiponectin, via the IRS pathway down-regulates the expression of atherosclerotic and angiogenic genes via AMPK. Few studies have investigated the effect of these counter-regulatory peptides in combination.

Leptin plays a role in matrix remodeling as it influences the expression of TIMP and MMP genes (Sweeney 2010). The TIMP genes are natural inhibitors or regulators of the effect of MMPs which help to break down and remodel tissues in the cardiovascular system (and other systems) by making sure the structure of tissues are maintained and that excess tissues collagen matrices are broken down and that they do not accumulate. Derangements in the expression of the genes responsible for the remodeling may lead to accumulation of collagen matrices and excess blood vessel formation (angiogenesis) leading to atherosclerosis and other cardiovascular diseases and even cancer (Park *et al.*, 2001).

Adiponectin on the other hand has been reported to have an opposite effect. It is however not completely clear how Leptin and adiponectin carry out their roles.

The aim of this study is to explore the role of leptin and adiponectin in the expression of these genes and to explore the various pathways in order to better understand the mechanism by which leptin and adiponectin have these effects on the expression of the genes. Also, the combined effect of the adipokines has been poorly researched and poorly understood and so this study aimed to explore the effect of combining leptin and adiponectin and to find out what the basis for the effects seen on the gene expression is.

3.2 Materials and Methods

The materials and the methods used for experiments that concern this chapter are detailed in the materials and method chapter (chapter 2). A summary of the process is stated as follows.

The procedure for investigating the effect of leptin and adiponectin on the expression of angiogenic genes required growing EAHY cells in 6 well plates and starving the endothelial cells of protein by placing them in 2% media for 6 hours to bring the cells to Go phase and then treating the cells with various concentrations of leptin and adiponectin in normal media (10%) while the controls were left untreated. This also involved adding AMPK, ERK and p38 inhibitors in the investigation of the bases of the effects of leptin and adiponectin. The AMPK inhibitor used was 10µg/ml Dorsomorphine Dihydrochloride from Tocris Bioscience Bristol, UK. The ERK inhibitor was ERK Inhibitor II, FR180204 and the p38 MAPK inhibitor was TAK 715 both from Santacruz Biomedicals, USA). The cells were then collected and RNA extracted, reverse transcribed and PCR carried out and the product run on a gel to find out what the role of AMPKI is on the expression of angiogenic genes MMP2, TIMP2 TIMP3.

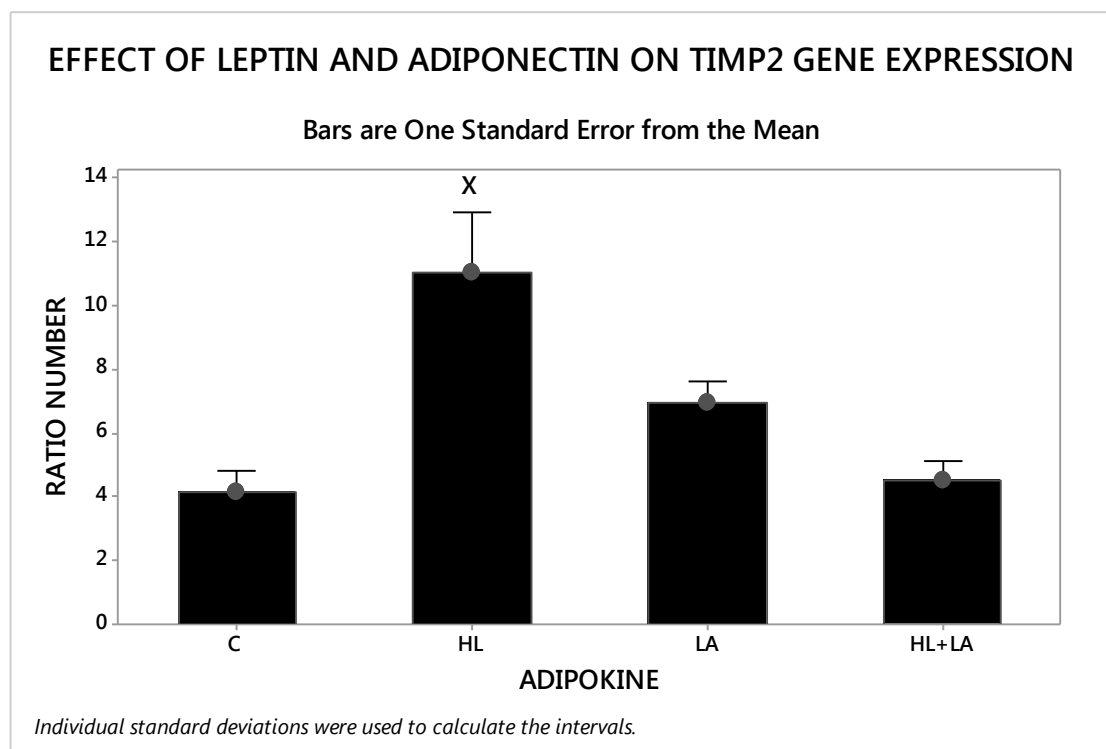
In Figures 3.1.1(a) and (b) to 3.1.3(a) and (b), (a) models the diabetic situation with high leptin (16ng/ml) and low adiponectin (36 ng/ml) while (b) represents the lean picture with High adiponectin (3.6µg/ml) and Low leptin (8ng/ml). All the cells used in the result below were grown in RPMI in 6 well plates, and serum starved, treated with adipokines and then the cells collected and RNA extracted from them. Total RNA was reverse-transcribed to cDNA and used to analyse gene expression by semi-quantitative PCR. Amplified products were run on 1% agarose gels and visualized under UV light to quantify the expression of each gene related to the amount of house-keeping gene (β -actin) present in the sample.

3.3 Results

3.3.1 Regulation of expression of genes that code for angiogenesis by individual and combined leptin and adiponectin treatments.

3.3.1.1 Effect of Leptin and Adiponectin on TIMP2

a)



b)

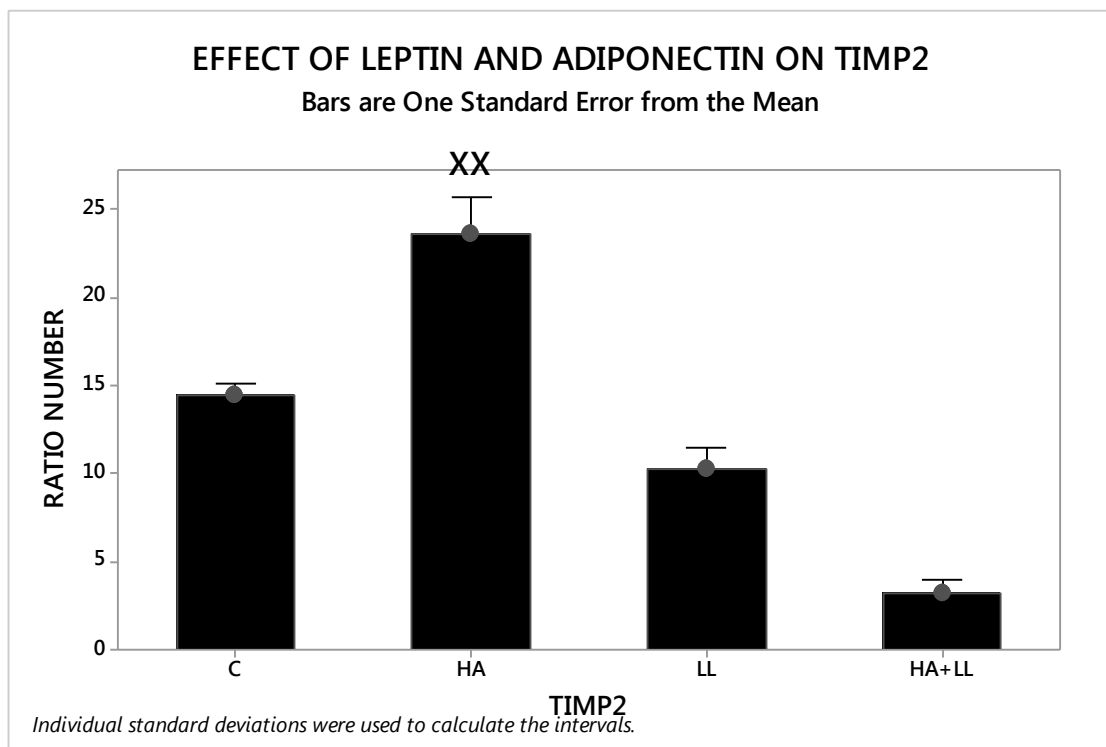
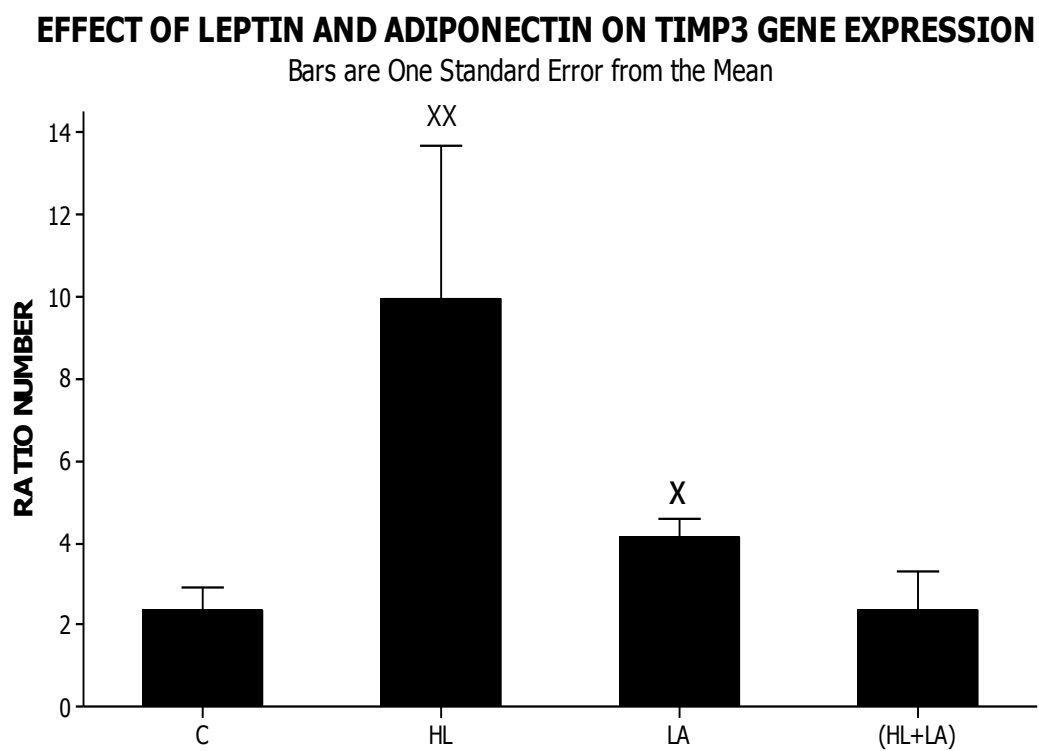


Figure 3.1.1 Effect of leptin and adiponectin individually and in combination on TIMP2 gene expression. (C- control, HL-high leptin, LL-low leptin, HA-high adiponectin, LA-low adiponectin, HL+LA- high leptin + low adiponectin, HA+LA- high adiponectin + low adiponectin)

Results from figure 3.1.1 (a and b) shows that leptin increases while Adiponectin reduces the expression of TIMP2. The results show that leptin significantly increased TIMP2 expression in high concentrations (3.1.1a) while adiponectin increases the expression of TIMP2 in High concentrations (3.1.1b). Also there is a significant reduction in the expression of the gene when leptin and adiponectin was combined (3.1.1 a and b). $p < 0.05$ using ANOVA and Tukey's multiple comparison tests for the comparison between untreated and leptin and adiponectin treated cells.

3.3.1.2 Effect of leptin and adiponectin on TIMP3 gene expression

a)



b)

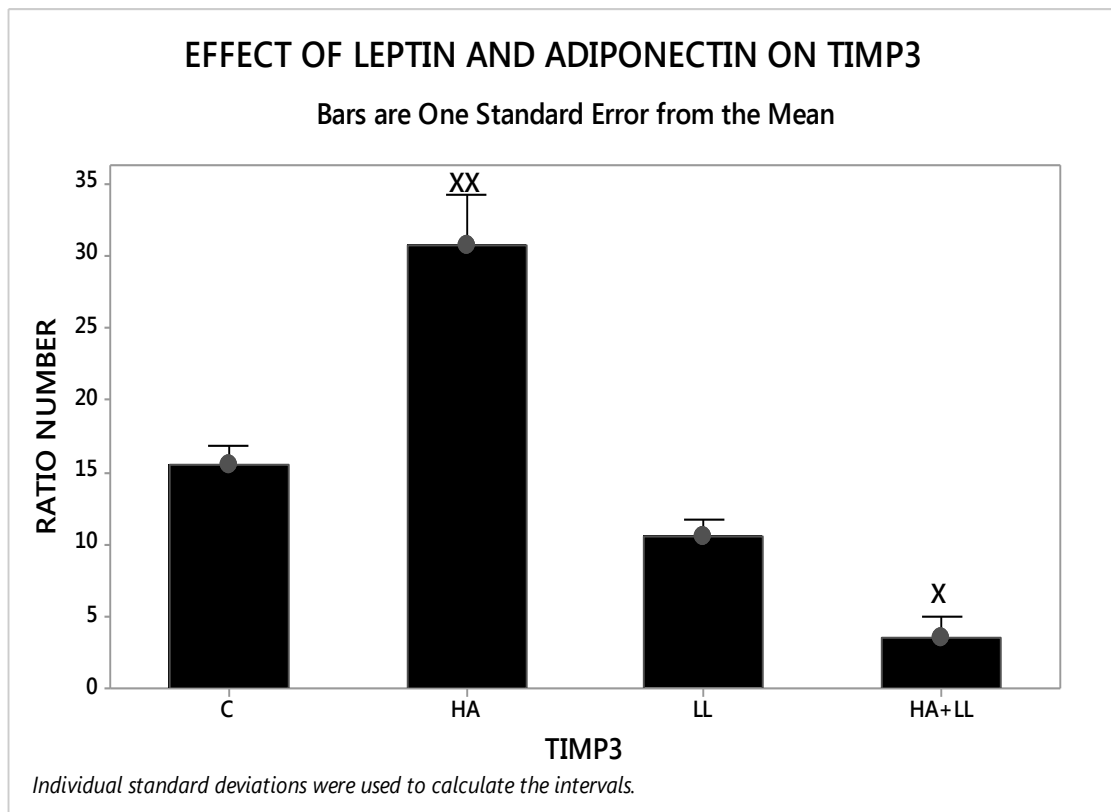


Figure 3.1.2 Effect of leptin and adiponectin individually and in combination on TIMP3 gene expression. (C- control, HL-high leptin, LL-low leptin, HA-high adiponectin, LA-low adiponectin, HL+LA- high leptin + low adiponectin, HA+LA- high adiponectin + low adiponectin)

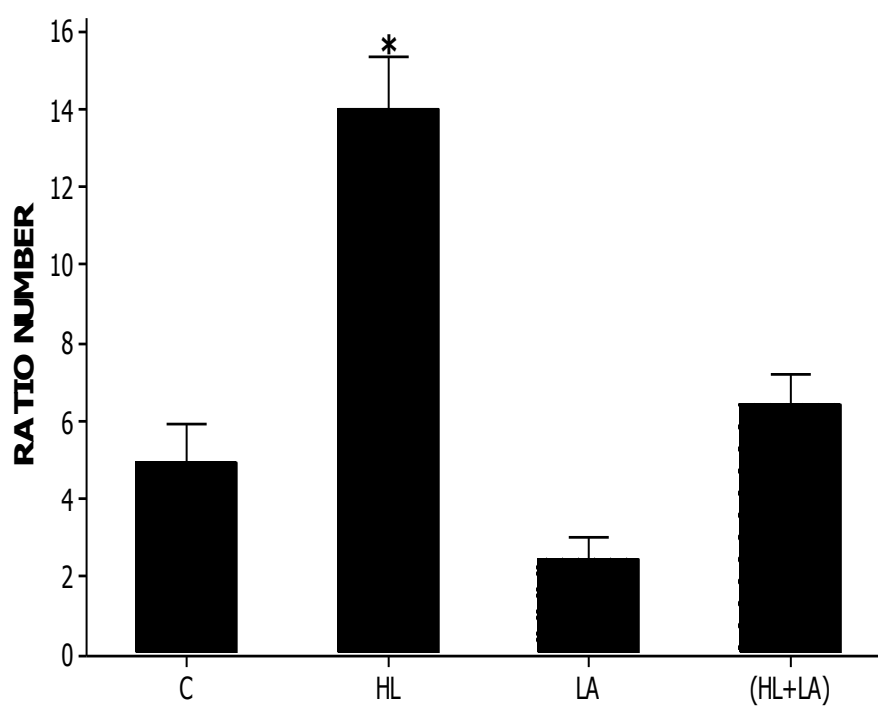
The results in figure 3.1.2 show that leptin increased TIMP3 expression in high concentrations (3.1.2a) while adiponectin increases the expression of TIMP3 in High concentrations (3.1.2b). Also there is a significant reduction in the expression of the gene when leptin and adiponectin was combined (3.1.2 a and b). $p < 0.05$ using ANOVA and Tukey's multiple comparison tests for the comparison between untreated and leptin and adiponectin treated cell.

3.3.1.3 Effect of leptin and adiponectin on MMP2 gene expression

a)

EFFECT OF LEPTIN AND ADIPONECTIN ON MMP2 GENE EXPRESSION

Bars are One Standard Error from the Mean



b)

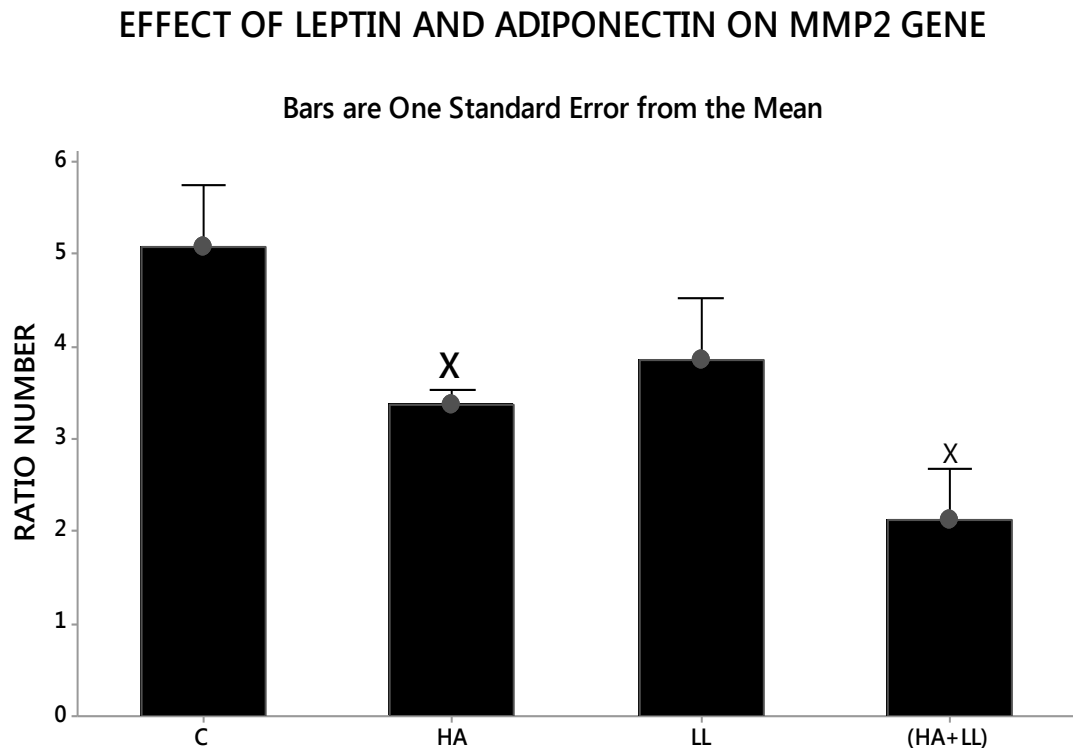


Figure 3.1.3 Effect of leptin and adiponectin individually and in combination on MMP2 gene expression. (C- control, HL-high leptin, LL-low leptin, HA-high adiponectin, LA-low adiponectin, HL+LA- high leptin + low adiponectin, HA+LA- high adiponectin + low adiponectin)

Figure 3.1.3 shows that leptin increases and adiponectin reduces the expression of MMP2 gene expression while combination of leptin and adiponectin down-regulates the expression of MMP2. The results from figures 3.1.3 (a and b) show that leptin increased MMP2 expression in high concentrations (3.1.3a) while adiponectin reduces the expression of TIMP3 in High and low concentrations (3.1.3b). Also there is a significant reduction in the expression of the gene when leptin and adiponectin was combined (3.1.3 a and b). $p < 0.05$ using ANOVA and Tukey's multiple comparison tests for the comparison between untreated and leptin and adiponectin treated cell.

3.3.2 Regulation of the effect of Leptin and adiponectin on angiogenic genes (MMP2, TIMP2, TIMP3) by AMPKI, ERKI and P38 inhibitor

3.3.2.1 The effect of 24 hour exposure of EAHY cells expression of Angiogenic genes to AM PKI in the presence of leptin and adiponectin

3.3.2.1.1 AMPKI has significant inhibitory effect on the effect of leptin on MMP2 gene expression

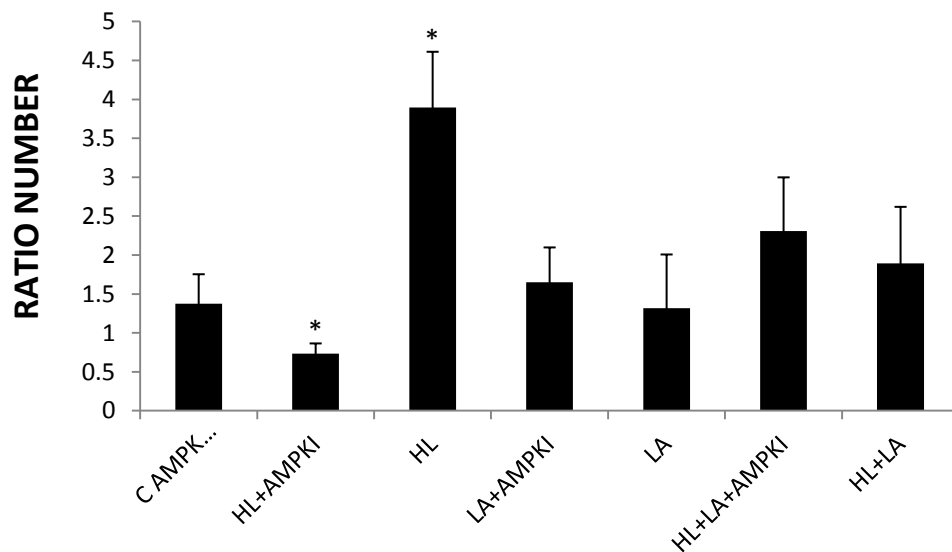


Fig 3.1.4 Role of AMPK in the effect of treating cells with leptin and adiponectin individually and in combination on the expression of MMP2 gene. (C AMPK MMP2- control, HL-high leptin, LA-low adiponectin, HL+LA-high leptin + low adiponectin, HA+LA- high adiponectin + low adiponectin, HL+AMPKI- high leptin + AMPK inhibitor, LA+AMPKI- low adiponectin + AMPK inhibitor, HL+LA+AMPKI- high leptin + low adiponectin + AMPK inhibitor)

Figure 3.1.4 shows that the increase of the expression of MMP2 by leptin (16ng/ml) is blocked significantly by AMPKI (10µg/ml). The inhibitory effect of low adiponectin (36ng/ml) on MMP2 was also blocked but not significantly. Adiponectin in low concentration reduced the effect of high leptin on MMP2 gene expression and blocking AMPK promotes the effect of leptin. P-value<0.05 using the students T-Test (2 way)

3.3.2.1.2 AMPKI significantly inhibits the effect of leptin on TIMP2 gene expression

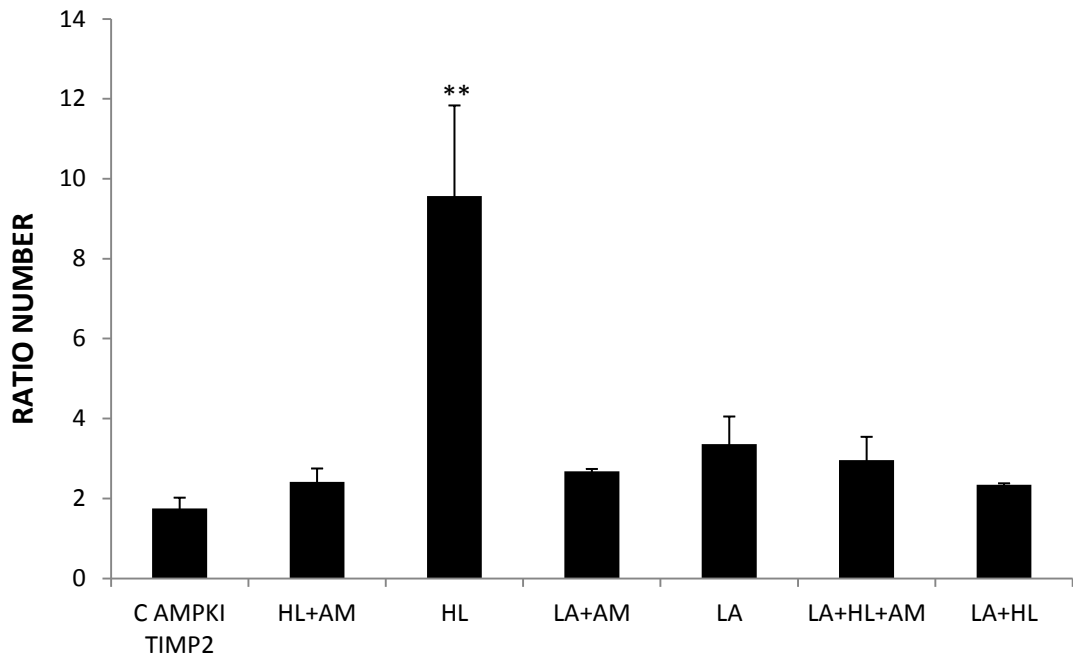


Fig 3.1.5 Role of AMPK in the effect of treating cells with leptin and adiponectin individually and in combination on the expression of TIMP2 gene. (C AMPK TIMP2- control, HL-high leptin, LA-low adiponectin, HL+LA- high leptin + low adiponectin, HA+LA- high adiponectin+ low adiponectin, HL+AMPKI- high leptin + AMPK inhibitor, LA+AMPKI- low adiponectin + AMPK inhibitor, HL+LA+AMPKI- high leptin + low adiponectin + AMPK inhibitor)

Figure 3.1.5 shows that AMPKI inhibits the effect of high leptin concentration on TIMP2 expression. The increase of the expression of TIMP2 by leptin (16ng/ml) is blocked significantly by AMPKI (10μg/ml). The effect of low adiponectin (36ng/ml) on TIMP2 was also blocked by AMPKI but not significantly. Adiponectin in low concentration reduced the effect of high leptin on TIMP2 gene expression and blocking AMPK promotes the effect of leptin. . P-value<0.05 using the students T-Test (2 way) to compare controls to samples.

3.3.2.1.3 AMPKI significantly inhibits the effect of leptin on TIMP3 gene expression

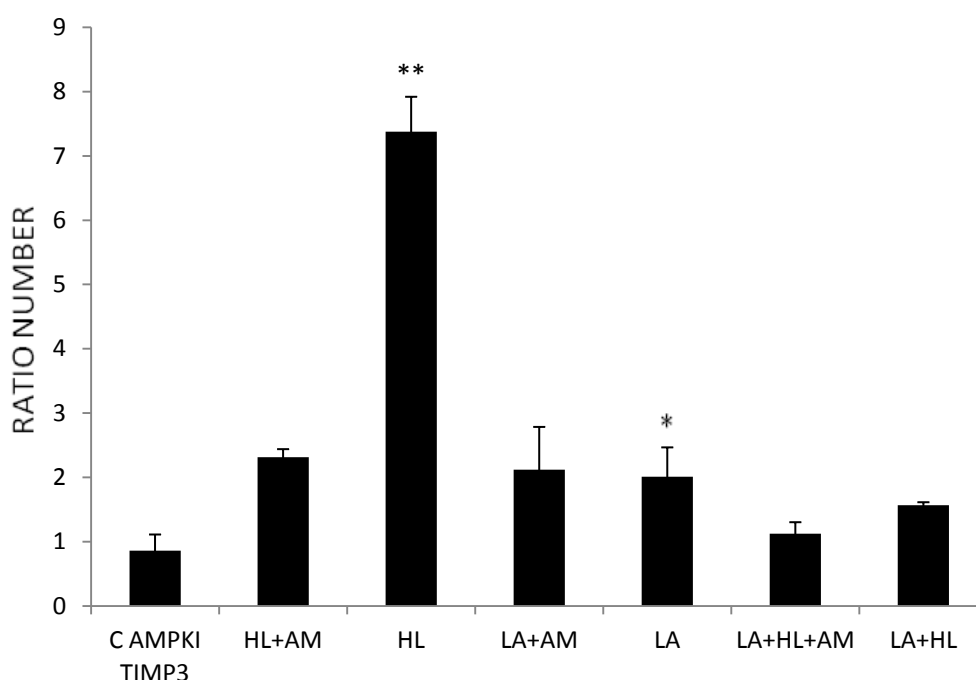


Fig 3.1.6 Role of AMPK in the effect of treating cells with leptin and adiponectin individually and in combination on the expression of TIMP3 gene. (C AMPK TIMP3- control, HL-high leptin, LA-low adiponectin, HL+LA- high leptin + low adiponectin, HA+LA- high adiponectin + low adiponectin, HL+AMPKI- high leptin + AMPK inhibitor, LA+AMPKI- low adiponectin + AMPK inhibitor, HL+LA+AMPKI- high leptin + low adiponectin + AMPK inhibitor)

Figure 3.1.6 shows that AMPKI inhibits the effect of high leptin concentration on TIMP3 expression. The increase of the expression of TIMP3 by leptin (16ng/ml) is blocked significantly by AMPKI (10µg/ml). The effect of low adiponectin (36ng/ml) on TIMP2 was not blocked by AMPKI. Adiponectin in low concentration reduced the effect of high leptin on TIMP3 gene expression and blocking AMPK inhibits the effect of the combination (not significantly). . P-value<0.05 using the students T-Test (2 way) to compare control to samples.

3.3.2.2 The effect of 24 hour exposure of EAHY cells expression of angiogenic genes to ERKI in the presence of leptin and adiponectin

3.3.2.2.1 ERKI significantly inhibits the effect of leptin and adiponectin on MMP2 expression

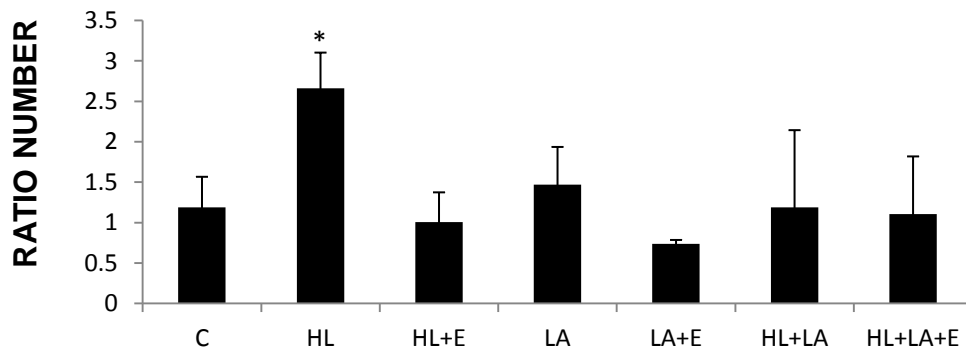


Figure 3.1.7 Role of ERK in the effect of treating cells with leptin and adiponectin individually and in combination on the expression of MMP2 gene. (C- control, HL-high leptin, LA-low adiponectin, HL+LA- high leptin + low adiponectin, HA+LA- high adiponectin + low adiponectin, HL+E- high leptin + ERK inhibitor, LA+E- low adiponectin + ERK inhibitor, HL+LA+E- high leptin + low adiponectin + ERK inhibitor)

Figure 3.1.7 shows that ERKI inhibits effect of high leptin and low adiponectin concentration on MMP2 expression. The increase of the expression of MMP2 by leptin (16ng/ml) is blocked significantly by ERK Inhibitor (10µg/ml). The effect of low adiponectin (36ng/ml) on TIMP2 was blocked significantly by ERKI. Adiponectin in low concentration reduced the effect of high leptin on MMP2 gene expression and blocking ERK does not promote the overall effect of leptin but reduces it. P-value<0.05 using the students T-Test (2 way) to compare control to samples.

3.3.2.2.2 ERKI significantly inhibits the effect of leptin and adiponectin on TIMP2 expression

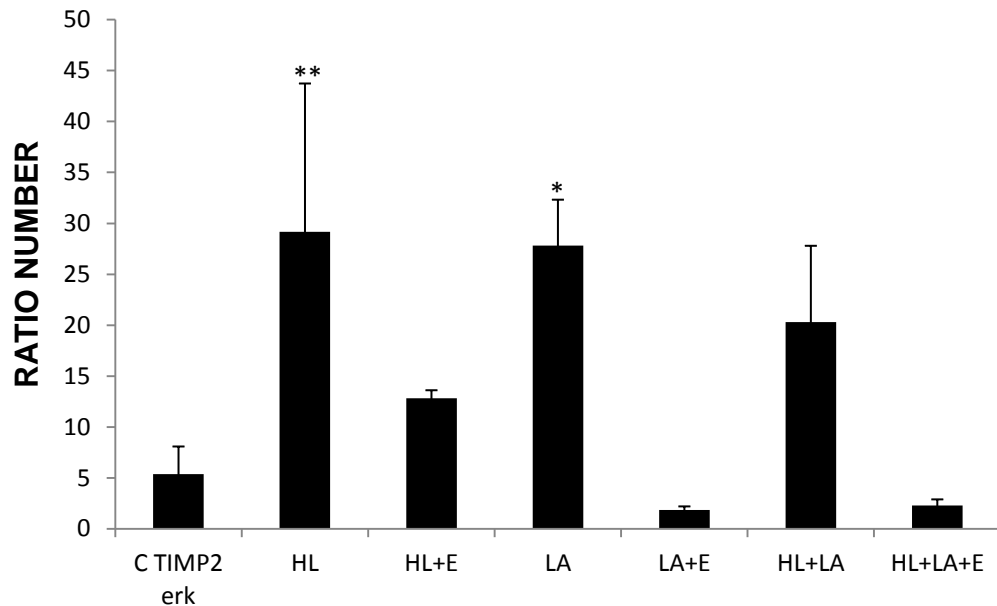


Figure 3.1.8 Role of ERK in the effect of treating cells with leptin and adiponectin individually and in combination on the expression of TIMP2 gene. (CTIMP2 ERK- control, HL-high leptin, LA-low adiponectin, HL+LA- high leptin + low adiponectin, HA+LA- high adiponectin + low adiponectin, HL+E- high leptin + ERK inhibitor, LA+E- low adiponectin + ERK inhibitor, HL+LA+E- high leptin + low adiponectin + ERK inhibitor)

Figure 3.1.8 shows that ERKI inhibits the effect of high leptin and low adiponectin concentration on TIMP2 expression. The increase of the expression of TIMP2 by leptin (16ng/ml) and adiponectin low adiponectin (36ng/ml) is blocked significantly by ERK inhibitor (10µg/ml). The effect of low adiponectin (36ng/ml) on TIMP2 was blocked significantly by ERKI. Adiponectin in low concentration reduced the effect of high leptin on TIMP2 gene expression (with an overall increase in gene expression) and blocking ERK in the leptin and adiponectin combination leads to an inhibition of TIMP2 expression. . P-value<0.05 using the students T-Test (2 way) to compare control to samples.

3.3.2.2.3 ERKI significantly inhibits the effect of leptin and adiponectin on TIMP3 expression

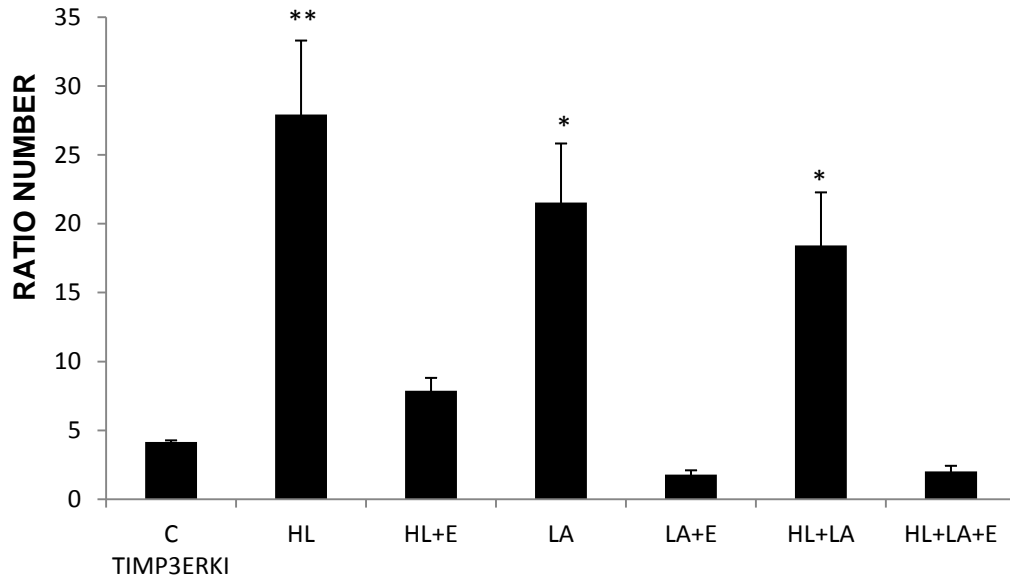


Figure 3.1.9 Role of ERK in the effect of treating cells with leptin and adiponectin individually and in combination on the expression of TIMP3 gene. (CTIMP3 ERKI- control, HL-high leptin, LA-low adiponectin, HL+LA- high leptin + low adiponectin, HA+LA- high adiponectin + low adiponectin, HL+E- high leptin + ERKinhibitor, LA+E- low adiponectin + ERK inhibitor, HL+LA+E- high leptin + low adiponectin + ERK inhibitor)

Figure 3.1.9 shows that ERKI inhibits the effect of high leptin and low adiponectin concentration on TIMP3 expression. The increase of the expression of TIMP3 by leptin (16ng/ml) and adiponectin low adiponectin (36ng/ml) is blocked significantly by ERK inhibitor (10µg/ml). Adiponectin in low concentration reduced the effect of high leptin on TIMP3 gene expression (with an overall increase in gene expression) and blocking ERK in the leptin and adiponectin combination leads to an inhibition of TIMP3 expression. . P-value<0.05 using the students T-Test (2 way) to compare controls to samples.

3.3.2.3 The effect of 24 hour exposure of EAHY cells to p38I on the expression of angiogenic genes in the presence of leptin and adiponectin

3.3.2.3.1 p38 Inhibitor inhibits the effect of leptin on MMP2 gene

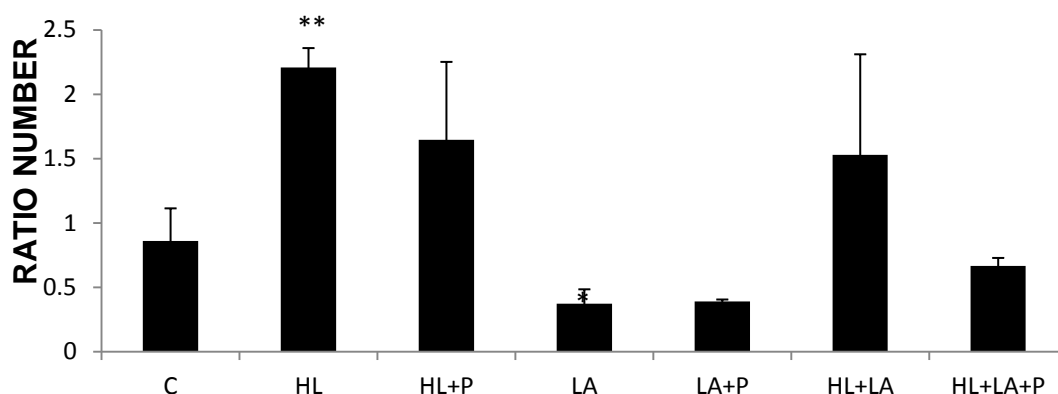


Figure 3.1.10 Role of p38 in the effect of treating cells with leptin and adiponectin individually and in combination on the expression of MMP2 gene. (C-Control, HL-high leptin, LA-low adiponectin, HL+LA- high leptin + low adiponectin, HA+LA- high adiponectin + low adiponectin, HL+P- high leptin + p38 inhibitor, LA+P- low adiponectin + p38 inhibitor, HL+LA+P- high leptin + low adiponectin + p38 inhibitor)

Figure 3.1.10 shows that P38I inhibits effect of high leptin and low adiponectin concentration on MMP2 expression. The increase of the expression of MMP2 by leptin (16ng/ml) is blocked by P38 inhibitor (10µg/ml). Adiponectin in low concentration (36ng/ml) reduced the effect of high leptin on MMP2 gene expression (with an overall increase in gene expression) and blocking p38 in the leptin and adiponectin combination leads to an inhibition of MMP2 expression. The effect of the adiponectin appears not to be affected by the p38I. . P-value<0.05 using the students T-Test (2 way) to compare control to sample.

3.3.2.3.2 P38I inhibits the effect of leptin and adiponectin on TIMP2 gene expression

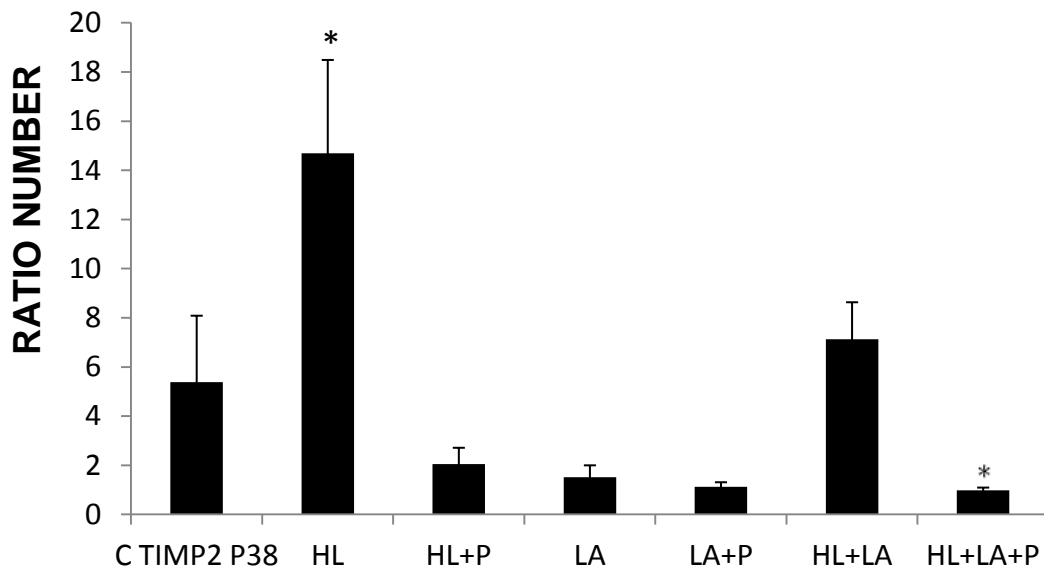


Figure 3.1.11 Role of p38 in the effect of treating cells with leptin and adiponectin individually and in combination on the expression of TIMP2 gene. (CTIMP2 p38-Control, HL-high leptin, LA-low adiponectin, HL+LA- high leptin + low adiponectin, HA+LA- high adiponectin + low adiponectin, HL+P- high leptin + p38 inhibitor, LA+P- low adiponectin + p38 inhibitor, HL+LA+P- high leptin + low adiponectin + p38 inhibitor)

Figure 3.1.11 shows that P38I inhibits the effect of high leptin on TIMP2 expression. The increase of the expression of TIMP2 by leptin (16ng/ml) is blocked by P38 inhibitor (10µg/ml). Adiponectin in low concentration (36ng/ml) reduced the effect of high leptin on TIMP2 gene expression (with an overall increase in gene expression) and blocking P38 in the leptin and adiponectin combination leads to an inhibition of TIMP2 expression ($p < 0.05$). The effect of the adiponectin is inhibited by the p38I. . P-value <0.05 using the students T-Test (2 way) to compare controls to samples.

3.3.2.3.2 p38I inhibits the effect of leptin and adiponectin on TIMP3 gene expression

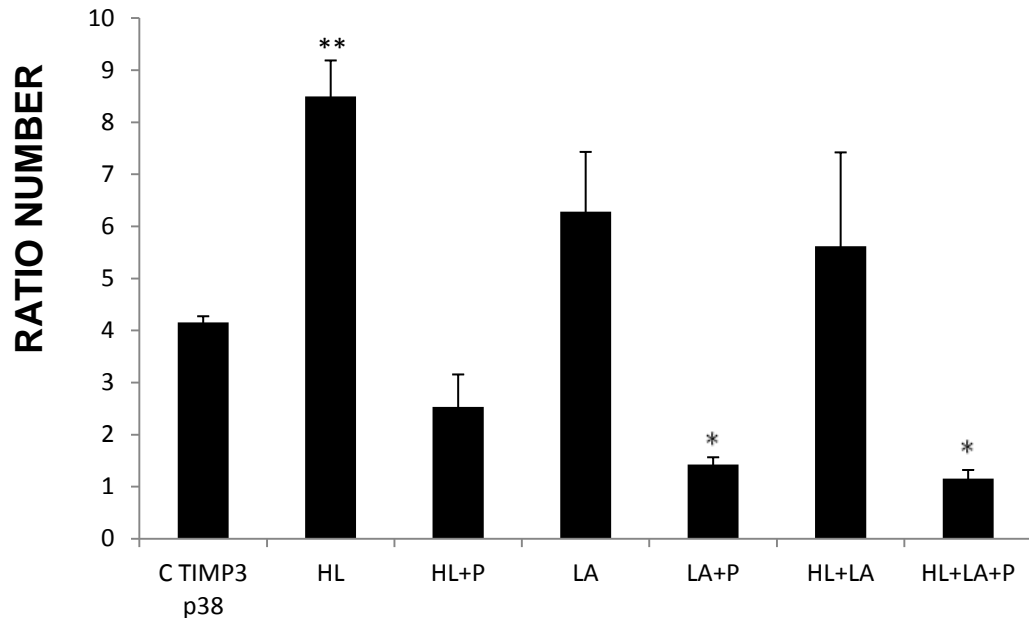


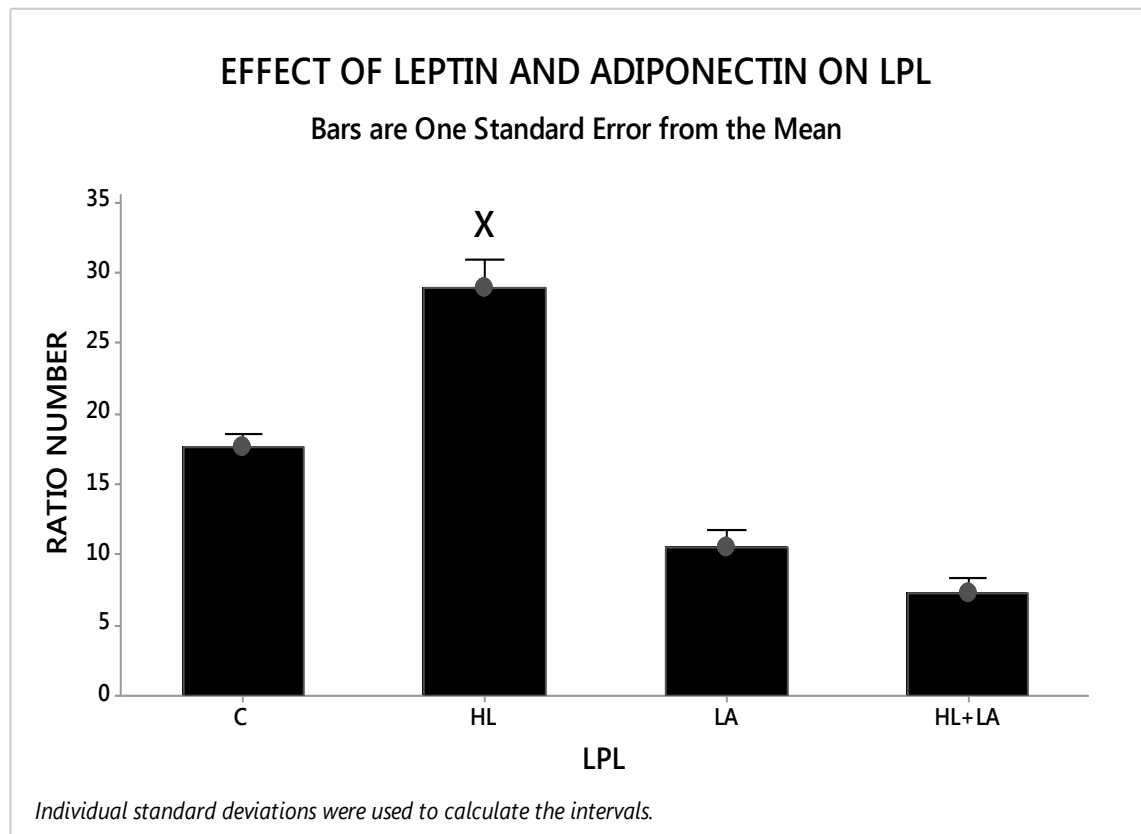
Figure 3.1.12 Role of p38 in the effect of treating cells with leptin and adiponectin individually and in combination on the expression of TIMP3 gene. (CTIMP3 p38-Control, HL-high leptin, LA-low adiponectin, HL+LA- high leptin + low adiponectin, HA+LA- high adiponectin + low adiponectin, HL+P- high leptin + p38 inhibitor, LA+P- low adiponectin + p38 inhibitor, HL+LA+P- high leptin + low adiponectin+ p38 inhibitor)

Figure 3.1.12 shows that P38I inhibits the effect of high leptin and low adiponectin on TIMP3 expression. The increase of the expression of TIMP3 by leptin (16ng/ml) and adiponectin (36ng/ml) is blocked by P38I (10µg/ml). Adiponectin in low concentration reduced the effect of high leptin on TIMP2 gene expression (with an overall increase in gene expression) and blocking P38 in the leptin and adiponectin combination leads to an inhibition of TIMP3 expression (significant). The effect of the adiponectin is inhibited by the p38I. . P-value<0.05 using the students T-Test (2 way) to compare control to sample.

3.3.3 Regulation of genes that code for atherosclerotic proteins by leptin and adiponectin individually and in combination

3.3.3.1 Effect of Leptin and adiponectin on LPL

a)



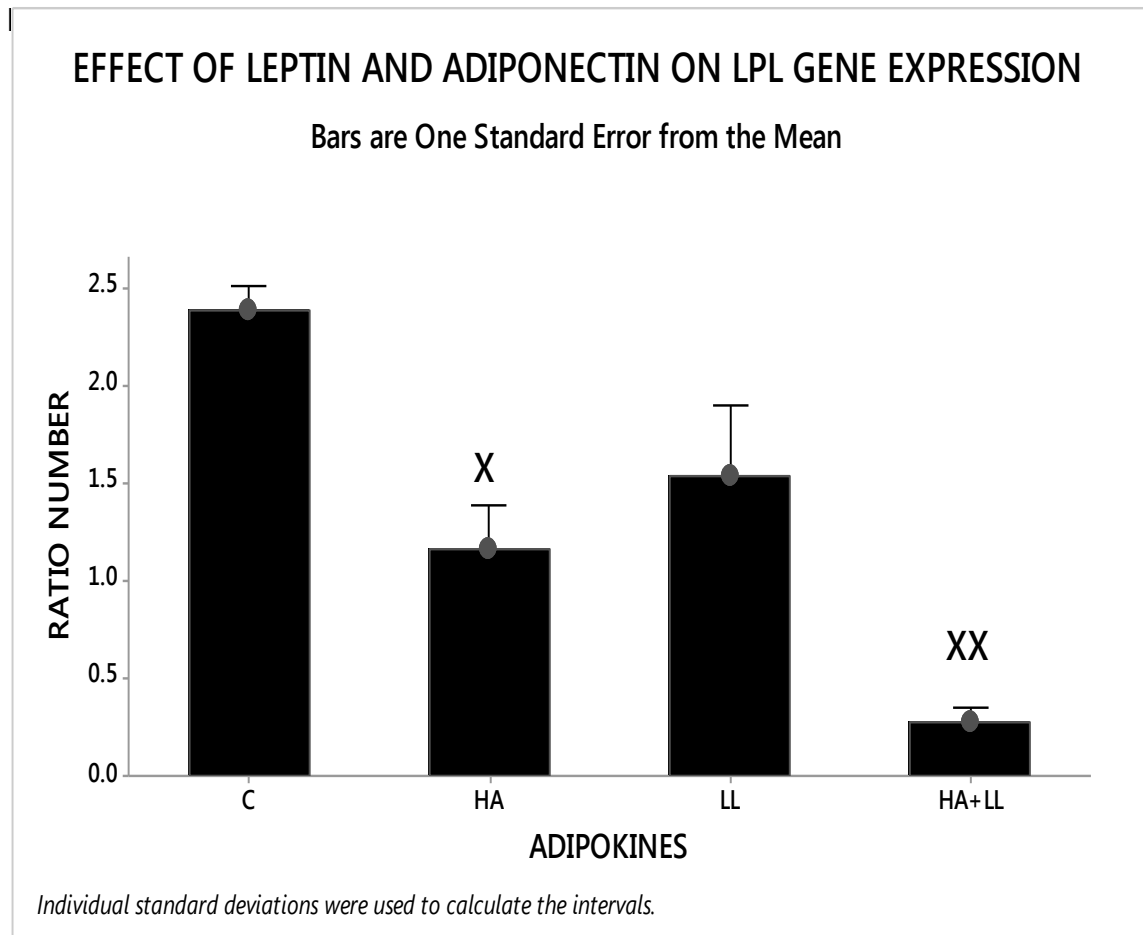
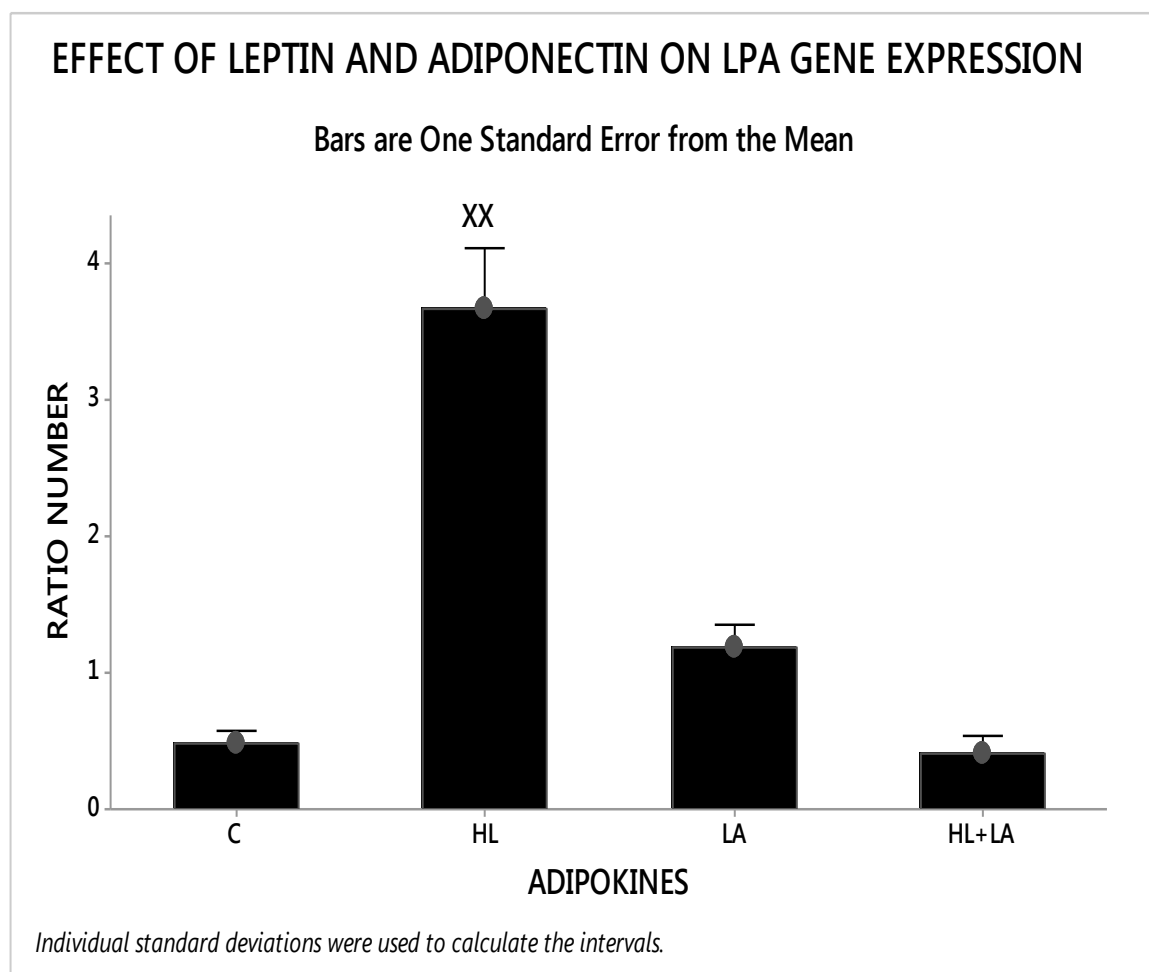


Figure 3.2.1 Effect of leptin and adiponectin individually and in combination on LPL gene expression. (C- control, HL-high leptin, LL-low leptin, HA-high adiponectin, LA-low adiponectin, HL+LA- high leptin + low adiponectin, HA+LA- high adiponectin + low adiponectin)

Figure 3.2.1 shows that leptin and adiponectin increases the expression of LPL gene expression while combination of leptin and adiponectin down-regulates the expression of LPL. The results show that Leptin increased LPL expression in high concentrations (3.2.1a) while adiponectin reduces the expression of LPA in High concentrations (3.2.1b). Also there is a significant reduction in the expression of the gene when leptin and adiponectin was combined (3.2.1 a and b). $p < 0.05$ using ANOVA and Tukey's multiple comparison tests for the comparison between untreated and Leptin and adiponectin treated cell.

3.2.2 Effect of Leptin and Adiponectin on LPA gene expression

a)



b)

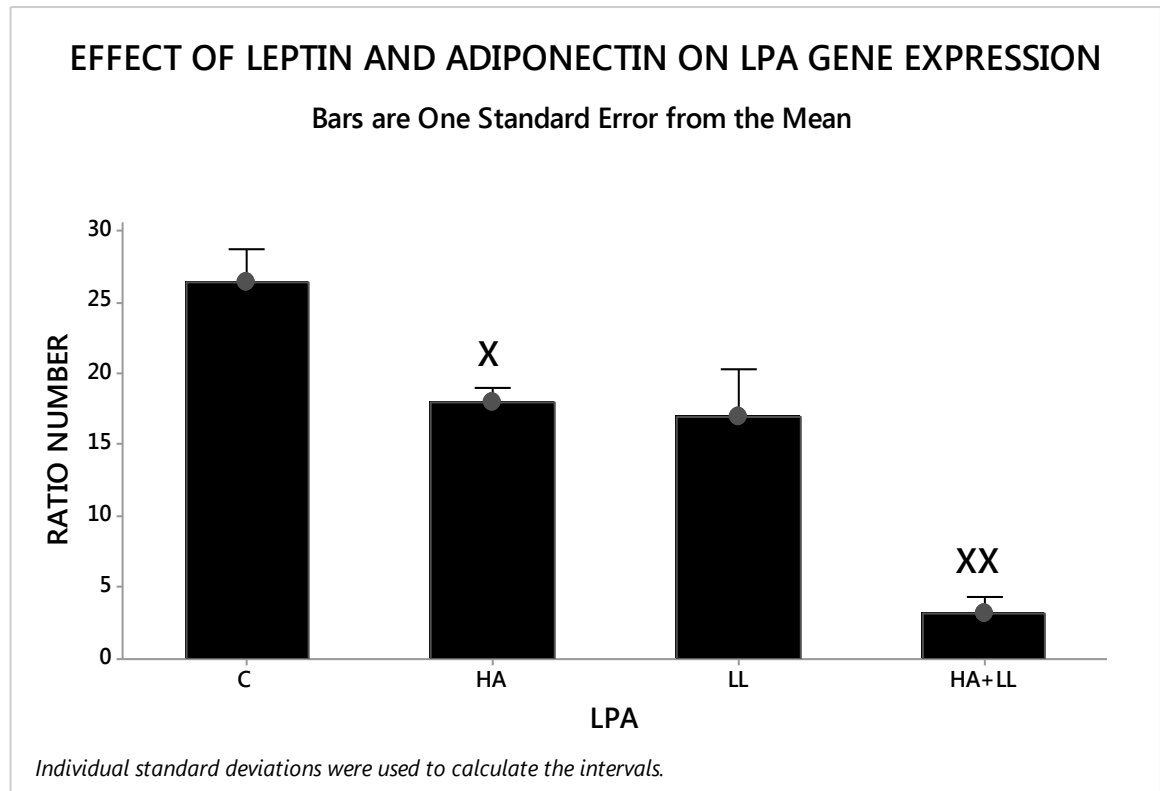


Figure 3.2.2 Effect of leptin and adiponectin individually and in combination on LPA gene expression. (C- control, HL-high leptin, LL-low leptin, HA-high adiponectin, LA-low adiponectin, HL+LA- high leptin + low adiponectin, HA+LA- high adiponectin + low adiponectin)

Figure 3.2.2 shows that leptin increases and adiponectin reduces the expression of LPL gene expression while combination of leptin and adiponectin down-regulates the expression of LPA. The results show that Leptin increased LPL expression in high concentrations (3.2.2a) while adiponectin reduces the expression of LPA in High and low concentrations (3.2.2b). Also there is a significant reduction in the expression of the gene when leptin and adiponectin was combined (3.2.2a and b). $p < 0.05$ using ANOVA and Tukey's multiple comparison tests for the comparison between untreated and Leptin and adiponectin treated cell.

3.3.4 The effect of 24 hour exposing EAHY cells to AMPKI in the presence of leptin and adiponectin on angiogenic gene

3.3.4.1 AMPKI inhibits the effect of leptin on LPA gene expression

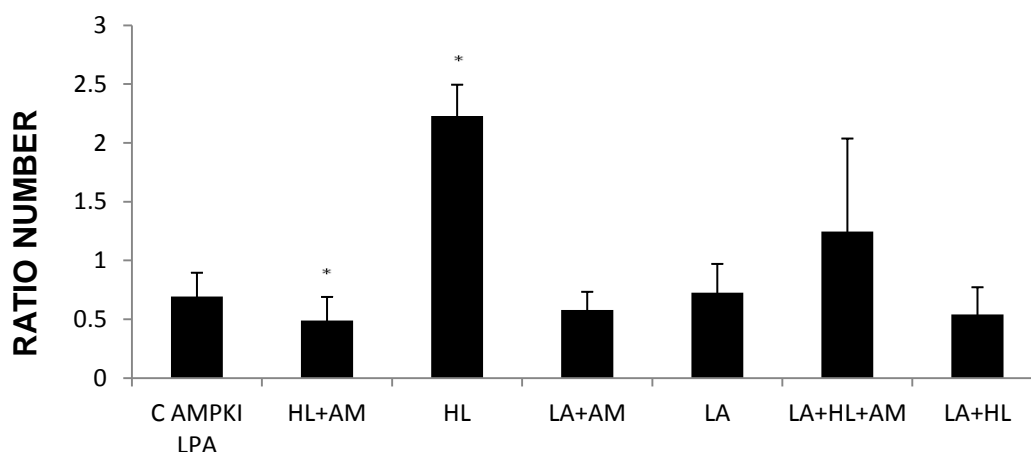


Figure 3.2.3 Role of p38 in the effect of treating cells with leptin and adiponectin individually and in combination on the expression of LPA gene. (C-AMPKI LPA-Control, HL-high leptin, LA-low adiponectin, HL+LA- high leptin + low adiponectin, HA+LA- high adiponectin + low adiponectin, HL+AM- high leptin+ AMPK inhibitor, LA+AM- low adiponectin + AMPK inhibitor, HL+LA+AM- high leptin + low adiponectin+ AMPK inhibitor)

Figure 3.2.3 shows that AMPKI inhibits the effect of high leptin concentration on LPA expression. The increase of the expression of LPA by leptin (16ng/ml) is blocked significantly by AMPKI (10µg/ml). The inhibitory effect of low adiponectin (36ng/ml) on LPA was also blocked but not significantly. Adiponectin in low concentration reduced the effect of high leptin on LPA gene expression and blocking AMPK promotes the effect of leptin. P-value<0.05 using the students T-Test (2 way) to compare control to sample.

3.3.4.2 ERKI inhibits the effect of leptin and adiponectin on LPA gene expression

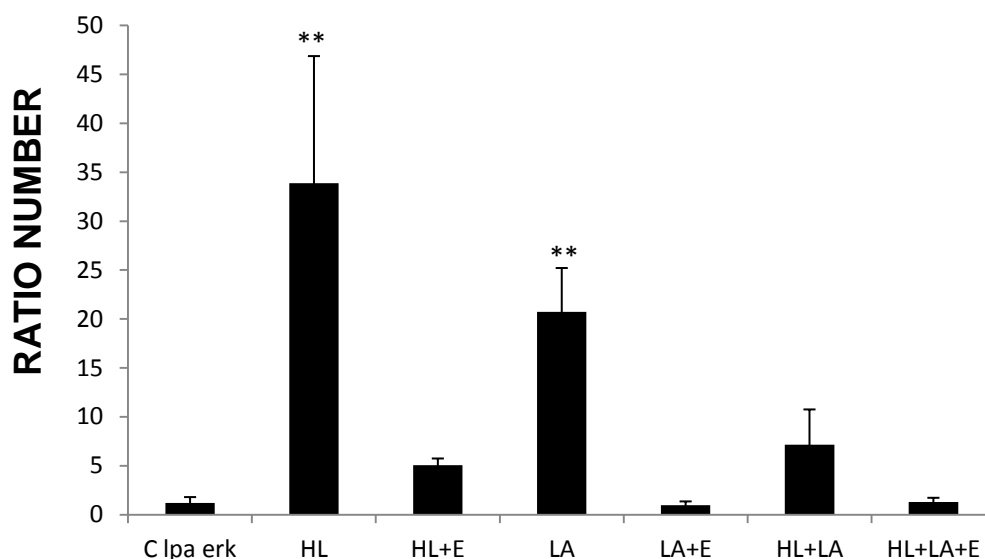


Figure 3.2.4 Role of ERK in the effect of treating cells with leptin and adiponectin individually and in combination on the expression of LPA gene. (CLPA ERK-Control, HL-high leptin, LA-low adiponectin, HL+LA- high leptin + low adiponectin, HA+LA- high adiponectin + low adiponectin, HL+E- high leptin + ERK inhibitor, LA+AM- low adiponectin + ERK inhibitor, HL+LA+E- high leptin + low adiponectin + ERK inhibitor)

ERKI inhibits effect of high leptin and adiponectin in LPA expression. The increase of the expression of LPA by leptin (16ng/ml) and adiponectin is blocked significantly by ERKI (10µg/ml). Adiponectin in low concentration (36ng/ml) reduced the effect of high leptin on LPA gene expression and blocking ERK promotes inhibition of LPA in the leptin and adiponectin combination. P-value<0.05 using the students T-Test (2 way) to compare control to sample.

3.3.4.3 p38I inhibits the effect of leptin and adiponectin on LPA gene expression

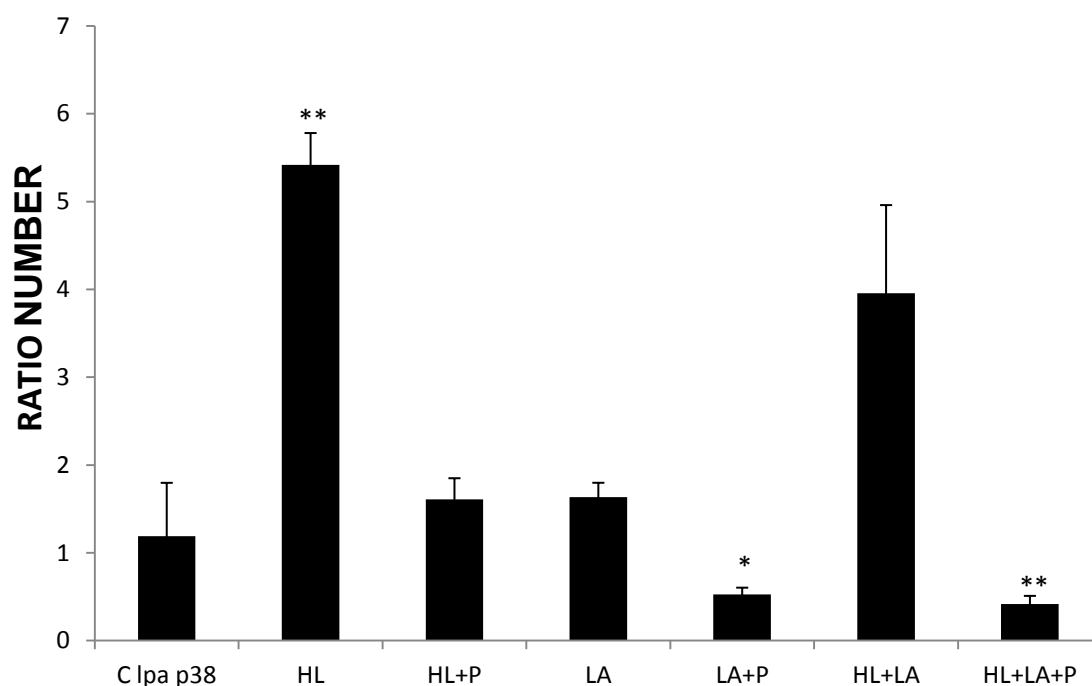


Figure 3.2.5 Role of p38 in the effect of treating cells with leptin and adiponectin individually and in combination on the expression of LPA gene. (CLPA p38-Control, HL-high leptin, LA-low adiponectin, HL+LA- high leptin + low adiponectin, HA+LA- high adiponectin + low adiponectin, HL+p- high leptin + p38 inhibitor, LA+P- low adiponectin + p38 inhibitor, HL+LA+P- high leptin + low adiponectin + P38 inhibitor)

Figure 3.2.5 shows that p38I inhibits effect of high leptin and adiponectin in LPA expression. The increase of the expression of LPA by leptin (16ng/ml) and adiponectin is blocked significantly by p38 inhibitor (10μg/ml). Adiponectin in low concentration (36ng/ml) reduced the effect of high leptin on LPA gene expression and blocking p38 promotes inhibition of LPA in the leptin and adiponectin combination. P-value<0.05 using the students T-Test (2 way) to compare control to sample.

3.3.5 Effect of p38 inhibitor and ERK inhibitor on MMP2,TIMP2,TIMP3 gene expression: further review with real time PCR

Though the expression of the genes has been explored by reverse transcription PCR, it was important to carry out a real time PCR in order to more accurately measure the expression of the genes expressed in order to more qualitatively assess the gene expression. The genes were well expressed with a Ct value of between 23 and 25.

3.3.5.1 Effect of P38 inhibitor on MMP2, TIMP2, TIMP3 gene expression

3.3.5.1.1 Effect of P38 inhibitor on MMP2 gene expression

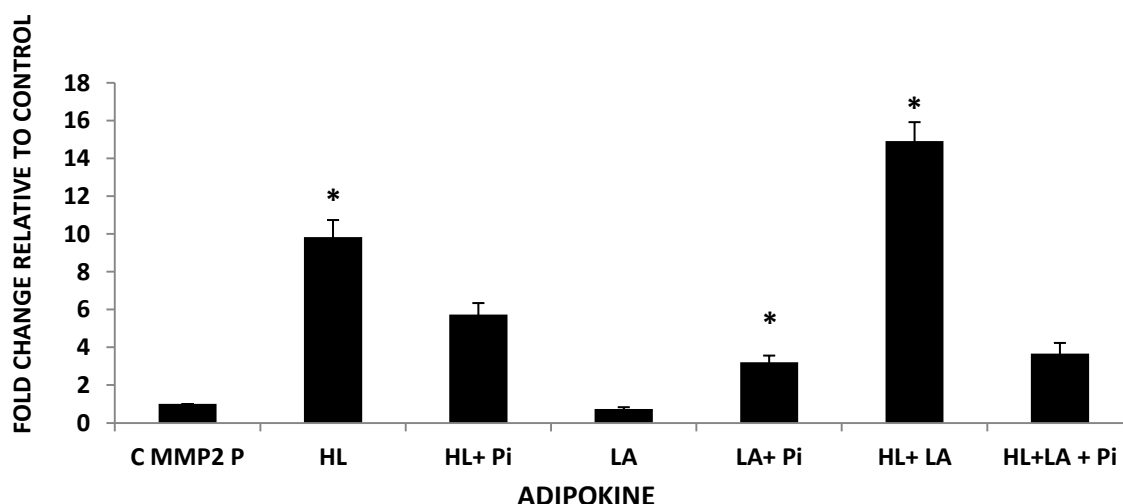


Figure 3.2.6 Role of p38 in the effect of treating cells with leptin and adiponectin individually and in combination on the expression of MMP2 gene. (CMMP2 P-Control, HL-high leptin, LA-low adiponectin, HL+LA- high leptin + low adiponectin, HA+LA- high adiponectin + low adiponectin, HL+P high leptin + p38 inhibitor, LA+P- low adiponectin + p39 inhibitor, HL+LA+P- high leptin + low adiponectin + p38 inhibitor)

Figure 3.2.6 shows the effect of p38 inhibitor on MMP2 gene expression. There is a significant increase in the MMP2 gene expression by the high leptin (HL-16ng/ml) which is reduced by the P38 inhibitor (10µg/ml). Low adiponectin (36ng/ml) reduced the expression of the MMP2 gene and this effect was blocked by the P38i. The combination of high leptin and low adiponectin (HL+LA) lead to a significant increase in the MMP2 gene expression which was reduced by the p38i. These results (from the real-time PCR) are equivalent to those obtained in the semi-quantitative PCR analysis. P-value<0.05 using the students T-Test (2 way) to compare control to sample.

3.3.5.1.2 Effect of P38 inhibitor on TIMP2 gene expression

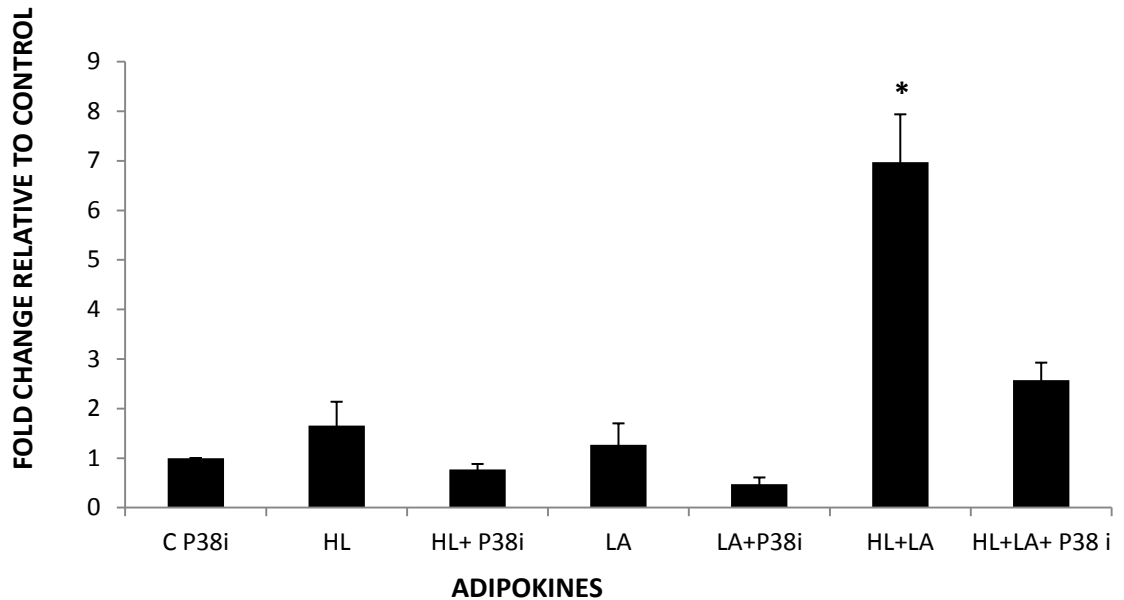


Figure 3.2.7. Role of p38 in the effect of treating cells with leptin and adiponectin individually and in combination on the expression of TIMP2 gene. (CP38i -Control, HL-high leptin, LA-low adiponectin, HL+LA- high leptin + low adiponectin, HA+LA- high adiponectin + low adiponectin, HL+P38i- high leptin + p38 inhibitor, LA+P38i- low adiponectin + p38 inhibitor, HL+LA+P38i- high leptin + low adiponectin + p38 inhibitor)

Figure 3.2.7 shows that there is an increase in the TIMP2 gene expression by the high leptin (HL-16ng/ml) which is reduced by the P38 inhibitor (10µg/ml). Low adiponectin (36ng/ml) significantly increased the expression of the TIMP2 gene and this effect was blocked by the P38i. The combination of high leptin and low adiponectin (HL+LA) lead to a significant increase in the TIMP2 gene expression which was reduced by the p38i. P-value<0.05 using the students T-Test (2 way) to compare control to sample.

3.3.5.1.3 Effect of p38 inhibitor on TIMP3 gene expression

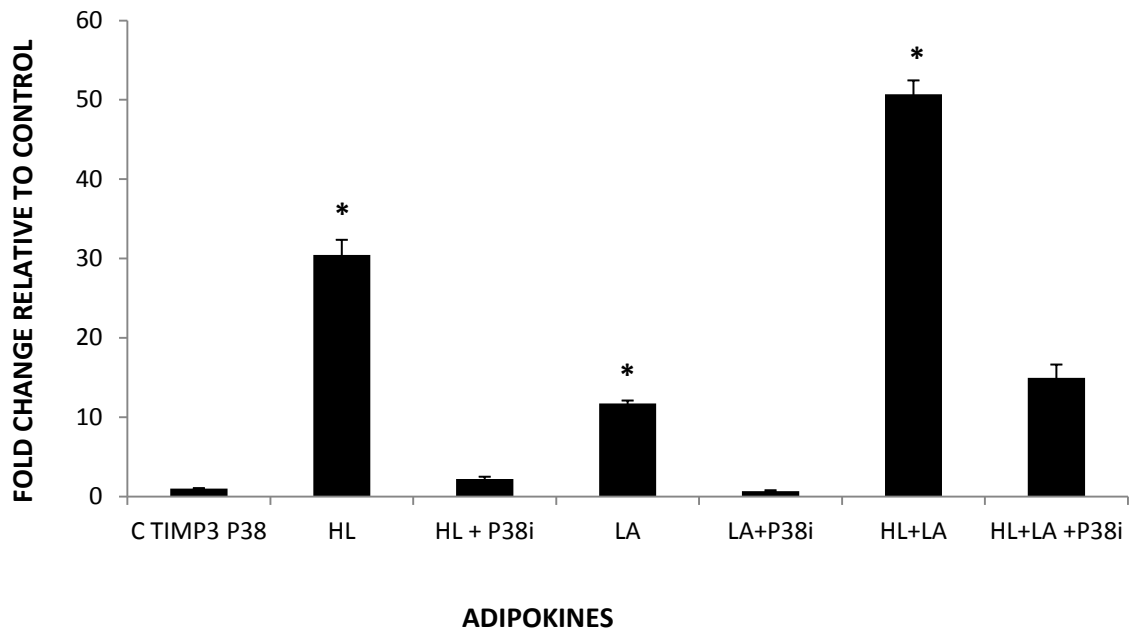


Figure 3.2.8. Role of p38 in the effect of treating cells with leptin and adiponectin individually and in combination on the expression of TIMP3 gene. (CTIMP3 P38-Control, HL-high leptin, LA-low adiponectin, HL+LA- high leptin + low adiponectin, HA+LA- high adiponectin + low adiponectin, HL+P38i high leptin + p38 inhibitor, LA+P38i- low adiponectin + p38 inhibitor, HL+LA+P38i- high leptin + low adiponectin + p38 inhibitor)

Figure 3.2.8 shows the effect of p38 inhibitor on TIMP3 gene expression. There is a significant increase in the TIMP3 gene expression by the high leptin (HL- 16ng/ml) which is reduced by the P38 inhibitor (10µg/ml). Low adiponectin (36ng/ml) increased the expression of the TIMP3 gene and this effect was blocked by the P38i. The combination of high leptin and low adiponectin (HL+LA) lead to a significant increase in the TIMP3 gene expression which was reduced by the p38i. P-value<0.05 using the students T-Test (2 way) to compare control to sample.

3.3.5.2 Effect of ERK inhibitor on MMP2, TIMP2 and TIMP3 gene expression

3.3.5.2.1 Effect of ERK inhibitor on MMP2 gene expression

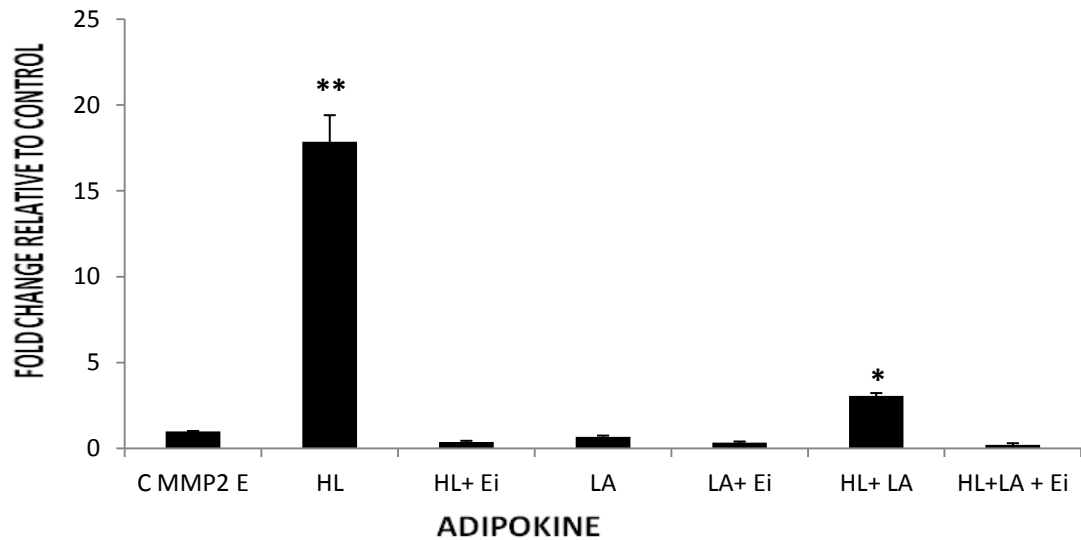


Figure 3.2.9. Role of ERK in the effect of treating cells with leptin and adiponectin individually and in combination on the expression of MMP2 gene. (CMMP2 E-Control, HL-high leptin, LA-low adiponectin, HL+LA- high leptin + low adiponectin, HA+LA- high adiponectin + low adiponectin, HL+Ei high leptin + ERK inhibitor, LA+Ei- low adiponectin + ERK inhibitor, HL+LA+Ei- high leptin + low adiponectin + ERK inhibitor)

Figure 3.2.9 shows the effect of ERK inhibitor on MMP2 gene expression. There is a significant increase in the MMP2 gene expression by the high leptin (HL- 16ng/ml) which is reduced by the ERK inhibitor (10µg/ml). Low adiponectin (36ng/ml) reduced the expression of the MMP2 gene and this effect was blocked by the ERKI. The combination of high leptin and low adiponectin (HL+LA) lead to a significant increase in the MMP2 gene expression which was reduced by the ERKI P-value<0.05 using the students T-Test (2 way) to compare control to sample.

3.3.5.2.2 Effect of ERK inhibitor on TIMP2 gene expression

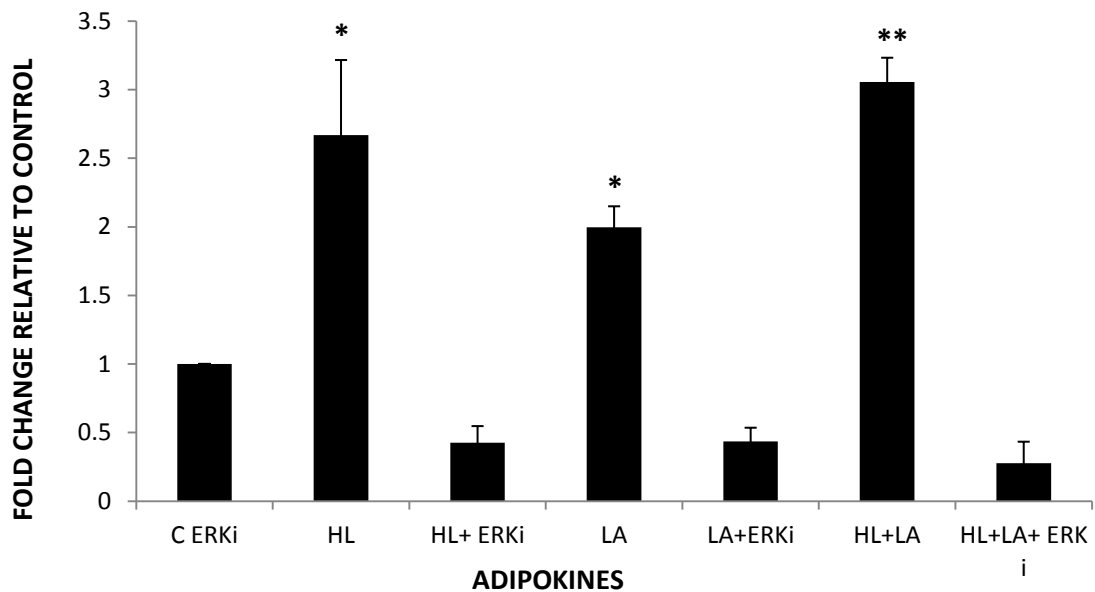


Figure 3.2.10. Role of ERK in the effect of treating cells with leptin and adiponectin individually and in combination on the expression of TIMP2 gene. (CERKi -Control, HL-high leptin, LA-low adiponectin, HL+LA- high leptin + low adiponectin, HA+LA- high adiponectin + low adiponectin, HL+ERKi high leptin + ERK inhibitor, LA+ERKi- low adiponectin + ERK inhibitor, HL+LA+ERKi- high leptin + low adiponectin + ERK inhibitor)

Figure 3.2.10 shows that there is a significant increase in the TIMP2 gene expression by the high leptin (HL-16ng/ml) which is reduced by the ERK inhibitor (10µg/ml). Low adiponectin (36ng/ml) increased the expression of the TIMP2 gene and this effect was blocked by the ERKI. The combination of high leptin and low adiponectin (HL+LA) lead to a significant increase in the TIMP2 gene expression which was reduced by the ERKI. P-value<0.05 using the students T-Test (2 way) to compare control to sample.

3.3.5.2.3 Effect of ERK inhibitor on the expression of TIMP3 gene

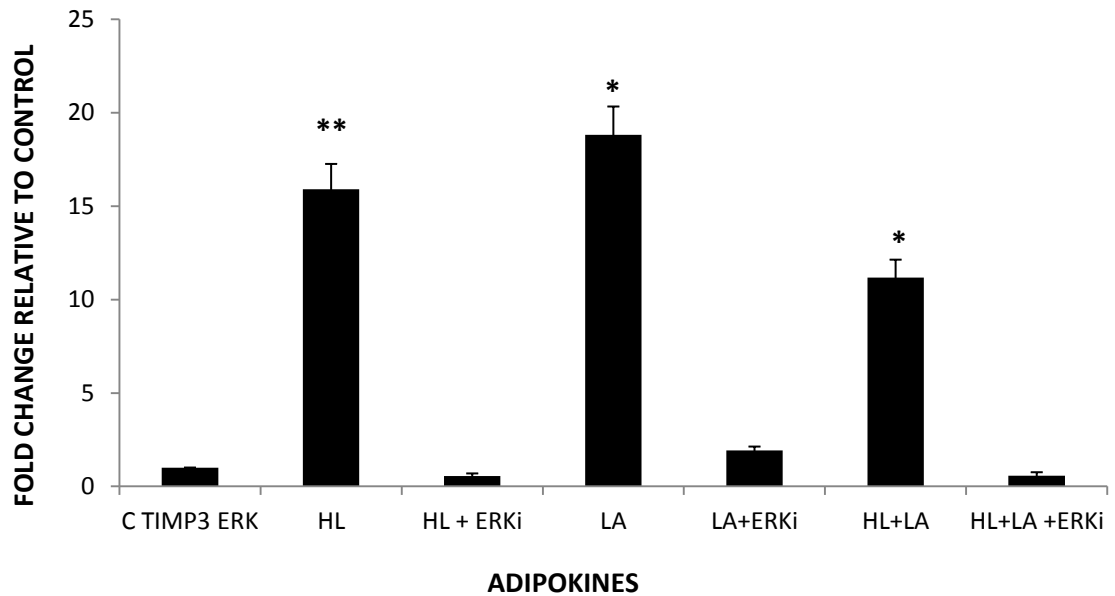


Figure 3.2.11. Role of ERK in the effect of treating cells with leptin and adiponectin individually and in combination on the expression of TIMP3 gene. (C TIMP3 ERK -Control, HL-high leptin, LA-low adiponectin, HL+LA- high leptin + low adiponectin, HA+LA- high adiponectin + low adiponectin, HL+ERKi high leptin + ERK inhibitor, LA+ERKi- low adiponectin + ERK inhibitor, HL+LA+ERKi- high leptin + low adiponectin + ERK inhibitor)

Figure 3.2.11 shows the effect of ERK inhibitor on TIMP3 gene expression. There is a significant increase in the TIMP3 gene expression by the high leptin (HL-16ng/ml) which is reduced by the ERK inhibitor (10µg/ml). Low adiponectin (36ng/ml) significantly increased the expression of the TIMP3 gene and this effect was blocked by the ERKi. The combination of high leptin and low adiponectin (HL+LA) lead to a significant increase in the TIMP3 gene expression which was reduced by the ERKi. P-value<0.05 using the students T-Test (2 way) to compare control to sample.

3.4 Discussion

Leptin and adiponectin are adipokines secreted by visceral fat and have been suggested to be links between obesity and cardiovascular diseases (Balistreri *et al.*, 2010). Obese individuals have been shown to have high levels of leptin (due to leptin resistance) and low levels of adiponectin and this is said to predispose to CVD. This is because leptin, which has been associated with an increase in the risk of CVD is at higher than normal levels, and adiponectin which has been linked with a reduction in the risk of CVD is at lower than normal levels (Dubey and Hesong 2006). Previous studies have shown a reverse of this in lean individuals (Klaus 2001). Also it has been shown that leptin increases the risk for CVD by increasing the expression of atherosclerotic and angiogenic genes (Park *et al.*, 2001, Koh *et al.*, 2008,), while adiponectin reduces the risk for CVD by reducing the expression of atherosclerotic and angiogenic genes and also reducing inflammation (Mattu and Randeva 2012). Adiponectin has also been suggested to reduce the risk of leptin causing CVD by inhibiting its effect on the expression of angiogenic and atherosclerotic genes (Zhang *et al.*, 2012). The mechanisms responsible for this are still not completely understood.

In the present study, the results in the experiments with endothelial cells suggest that leptin increases the risk of CVD while adiponectin reduces it and various pathways have been explored in order to better understand the reasons why the results are so. This study provides novel insights into the basis of the

effects and also explores the effect of the adipokines when combined which provides strong and interesting evidence that suggest that the risk of CVD predisposition by high leptin can be reduced by adiponectin.

Angiogenesis which is the formation of new blood vessels from pre-existing blood vessels is linked to atherosclerosis (formation of an atheromatous plaque in the blood vessels or heart) and both form most of the basis for CVD (Cao 2007). Studies have shown that CVD occurs when there is a derangement in the balance in the remodeling process carried out by MMP (Matrix Metalloproteinases) which break down excess tissues and TIMPs which inhibit MMPS, thereby ensuring just enough tissue formation in injuries and inflammatory processes within the blood vessels (can also lead to excess accumulation of collagen or extracellular matrix if there is an expression in excess) (Dollery *et al.*, 1995). In this study, the angiogenic genes that were expressed by the EAHY cells are TIMP2 and 3 (Tissue Inhibitor of Metalloproteinases 2 and 3) and MMP2 (Matrix Metallopeptidase 2). It must be noted that the TIMP in practical terms act as a balance to MMPs and TIMP can predispose to CVD (atherosclerosis) if there is over-expression without a corresponding amount of MMPs to inhibit, while the MMPs can lead to angiogenesis and excess expression can lead to weakening of blood vessels, and excess smooth muscle cell migration and macrophage activation leading to an atheromatous plaque (Dollery *et al.*, 1995).

In summary, the result from the present study shows that treating the cells with 16ng/ml of leptin had the maximal effect in upregulating the MMP2, TIMP2 and TIMP3 ($p<0.05$) while 3.6 μ g/ml of adiponectin caused a maximal down regulatory effect on MMP2 and an up-regulatory effect on TIMP2 and TIMP3 ($p<0.05$) compared to cells left untreated (controls). At high concentrations, adiponectin appears to be able to inhibit the increase in the expression of angiogenic genes, while at low concentration, adiponectin did not have any effect on the increase in the expression of the expressed angiogenic genes.

Leptin on the other hand appears to have an up-regulatory effect on the angiogenic genes at high concentration and at low concentration leptin showed no significant effect on increasing the expression of angiogenic genes (Figures 3.1.1- 3.1.3). With regards to atherosclerosis, results from this study shows that leptin increases while adiponectin reduces the expression of atherosclerotic genes and combining leptin and adiponectin causes a reduction in the expression of atherosclerotic genes when the concentration of adiponectin is high as against when the concentration of adiponectin is low. All these effects were also linked to the enzymes AMPK, ERK and p38. At 16ng/ml leptin increases LPL and LPA expression ($p<0.05$) while adiponectin at 36ng/ml had an inhibitory effect on the LPA and LPL and 3.6 μ g/ml increase the expression of LPA genes ($p<0.05$). A combination of the adipokines showed adiponectin at high concentrations inhibiting the expression of atherosclerotic genes while in low concentration, had no significant effect in reducing the effect of leptin in increasing the expression of atherosclerotic genes. The role of AMPK, ERK and

p38 in the effect of leptin and adiponectin was also explored (Figure 3.2.1-3.2.7).

The normal adiponectin level in humans is reported to be 3-14µg/ml while the human leptin concentration is between 5-15ng/ml in lean (Zabadi *et al.*, 2010) and 10-50ng/ml in obese (Kazmi *et al.*, 2013). In this study 3.6µg/ml and 36ng/ml of adiponectin were used as the high and low concentrations of adiponectin (HA, LA) while 16ng/ml and 8ng/ml were used as the high and low concentrations of leptin (HL, LL).

The experiments that gave rise to the results were designed to simulate what would be found in an obese diabetic or non-diabetic (HL+ LA) and lean person (HA+LL) (diabetic or non diabetic). It is therefore important to look at the results as a whole and not individually.

A general overview of the effect of leptin on the angiogenic genes in this study shows that leptin increases the expression of TIMP 2, 3 and MMP2 in high concentrations only and not in low concentrations. This shows a resultant increase of angiogenic genes which may predispose to CVD especially in conditions like obesity where leptin levels are very high. On the other hand, adiponectin in high and low concentrations increased the expression of all the TIMPs but inhibited the expression of MMP at all concentrations. This suggests

that adiponectin has a resultant effect of reducing MMP2 which contributes to plaque formation and increases the expression of TIMPS which inhibits MMPs (Hopps *et al.*, 2011). This therefore suggest a pattern that discourages plaque formation and slows down the formation of plaque by the breakdown of tissues which form plaque by the increase in TIMP leading to damage of vascular endothelial cells and embolus formation.

The combination of leptin (LL, HL) with adiponectin (HA, LA) shows a pattern of significant inhibition of angiogenic genes (TIMPs and MMPs) when the combination is HA+LL and that of a significant increase when HL+LA suggesting that when adiponectin is in a high enough concentration, it may have a neutralizing effect on the up-regulatory effect of leptin. Therefore looking more closely, with regards to angiogenesis, the results from this study suggests that leptin increases angiogenesis while adiponectin reduces it. Furthermore, a combination of leptin and adiponectin appear to reduce angiogenesis with evidence linking the involvement of AMPK, P38 and ERK enzymes in the effects of leptin and adiponectin.

In vitro studies on the effect of leptin on angiogenesis have shown that leptin increases the expression of TIMP 2, TIMP3 and MMP2 at concentrations of 10-40 ng/ml(Cao, 2007, Qi *et al* 2004, and Park *et al* 2001, Chen *et al* 2012, Madani *et al.*, 2005) thus suggesting that leptin increases the risk of angiogenesis. Therefore the data from the results in figure 3.1.1 to 3.2.11

strongly highlights the potential role of leptin in angiogenesis based on the significant ($P < 0.05$) up-regulation of the three genes by 16ng/ml (HL) of leptin. The results show that at 8ng/ml (LL), leptin had no significant effect on these genes. This view is in contrast to the studies carried out by Xia *et al* (2006) which was done on rodents and which suggested that leptin inhibits TIMP2 secretion. This highlights the variation in the results of *in-vivo* compared to *in-vitro* studies and could suggest the need to do more *in-vitro* studies in order to further investigate the effect of leptin on TIMP2.

As stated earlier, the basis of the effect of leptin on the angiogenic genes was investigated by exploring the role of AMPK (5' AMP- activated protein kinase), ERK (Extracellular signal-regulated kinase) and p38 (p38 mitogen-activated protein kinase). In the study by Li *et al.* (2005) and Madani *et al.* (2006), the effect of leptin on angiogenic genes was shown to be mediated through ERK 1 and 2 and p38 MAPK. Minikoshi and Khan (2003), however suggested that leptin mediates fatty acid oxidation in skeletal muscles via AMPK, thus inhibiting the effect of fat cells on insulin sensitivity (lipid toxicity), but very little is known about the role of AMPK in the effect of leptin in angiogenesis. The present study strongly supports the view that leptin mediates its action on angiogenic genes via ERK and p38 MAPK. It is also interesting to note that the result from this study suggest that AMPK is involved in the effect of leptin in the expression of angiogenic genes. This may be another area for further studies in the future.

The studies by Kim *et al.* (2009), Nakasone *et al.* (2013), Chen *et al.* (2012) and Zhu *et al.* (2008) suggested that adiponectin has vaso-protective qualities and showed that adiponectin increases the expression of TIMP2 and TIMP3 and inhibits the expression of MMP2 at adiponectin concentrations between 30ng/ml to 20µg/ml. It was suggested by Madani *et al.* (2005) that MMP2 is mainly responsible for breakdown of extracellular matrix as it is said to be able to activate other MMPs. The implication of this is that if it is expressed in excess, it is able to break down scars from damages to the heart causing ballooning of the heart and may also dislodge atheromatous plaques thus predisposing to embolism (Madani *et al.* 2005). TIMPs have been shown to balance this effect (Chen *et al.* 2012). The data in figure 3.1.1 – figure 3.2.11 (especially the effect of high adiponectin- 3.6µg/ml), provides further support to these studies by further providing a strong evidence to support the view that leptin is vaso protective. However, not all the previous studies agree with this view as studies by Adya *et al.* (2012) and Benaitrueau *et al.* (2010) have suggested that adiponectin increases the expression of MMP2 and reduces the expression of TIMP2. The difference in results may be due to the fact that it was the globular adiponectin (gAd) that had the up-regulatory effect on MMP2 unlike the full length (fAd) which is what is used in the present study and elicits an inhibitory effect on the expression of MMP2 and Beaitrueau *et al.* (2010) used low doses (25-250ng/ml) which is much lower than the adiponectin concentration that inhibited MMP2 in the present study.

Adiponectin has been shown to mediate its effects on angiogenic genes via AMPK (Kim *et al.*, 2009, Zhang *et al.*, 2012, Mattu and Randeva 2012), ERK 1 and 2 (Lee *et al.*, 2008). Adiponectin has been suggested to increase fatty acid oxidation in skeletal muscles via p38 MAPK. This process has been shown to reduce “lipotoxicity” and reduces insulin resistance in skeletal muscles (Yoon *et al.*, 2006). Very little has however been done to explore the role of p38 MAPK in the pathogenesis of angiogenesis and atherosclerosis. It is interesting to note that the result from this study does not support (with a significant result) the suggestion that AMPK mediates the effect of adiponectin on the angiogenic genes as the effect seen was inhibited in a few cases (but not significantly) and in some not inhibited at all by AMPK inhibitor. The reason for the difference may be as a result of the fact that some of the previous studies have used concentrations of AMPKI ranging from 1-100µg/ml (against 10µg/ml in the present study) in *in-vivo* and *in-vitro* experiments with varying conditions. The role of ERK in the effect of adiponectin on angiogenic genes was strongly highlighted in figures 3.1.7-3.1.9 where the up regulatory effect of adiponectin was significantly ($P<0.05$) blocked by the ERK inhibitor. The results in figures 3.1.10-3.1.12 suggests that p38MAPK may have a role in mediating the effect of adiponectin on angiogenic genes. Due to the fact that there are very few studies that have looked at this, the role of p38MAPK in angiogenesis in endothelial cells may need to be further explored in future research.

With regards to the role of leptin in atherosclerosis, the expression of LPA and LPL was explored. Studies by Maingrette and Reiner (2003), Hirata *et al.* (1999)

and Mead and Ramji (2001), suggested that LPL is not synthesized in endothelial cells, but in parenchymal cells such as adipocytes, muscle cells and macrophages. LPL in the plasma is said to be anti-atherogenic while LPL secreted by macrophages in the arterial wall promotes the retention of lipoproteins in the subendothelial space and favours the adhesion of monocytes to the endothelium and the transformation of these monocytes to macrophages which further attracts more monocytes by the secretion of more LPL (Dubey and Hesong 2006). In these tissues however, it was shown that a concentration of 10nM of leptin (as against 1nM used in the present study) is able to increase the expression of LPL. Results from the present study provide evidence to show that the LPL gene is expressed by endothelial cells as against the view from previous studies that suggest that LPL is produced only by parenchymal cells.

LPA (Lipoprotein a) has been suggested to be a risk factor for atherosclerosis and the basis of this has been explained to be due to similarities in structure between LPA and plasminogen. LPA binds to plasminogen activator receptor and interrupts the effect of plasminogen activator which converts plasminogen to plasmin thereby inhibiting the formation of plasmin, and therefore the breakdown of fibrin by plasmin leading to accumulation of fibrin and an increase in the risk of atherosclerosis (Buechler *et al.*, 2001). The result from the present study therefore highlights the possible risk of leptin being an atherosclerotic factor as the result shows that 1nM leptin caused a significant up-regulation in LPA gene expression in endothelial cells. Therefore, result in figure 3.2.1

provides evidence that suggest that leptin at 1nM increases LPA gene expression.

The experiments which form the basis of the results shown in figure 3.2.3 -3.2.5 explore the possibility of AMPK, ERK and p38 MAPK forming the basis of the effect of leptin on LPA. The result suggest that the up-regulatory effect of leptin on LPA is mediated by AMPK, ERK and p38 MAPK. Most of the previous studies on the effect of leptin on LPA and LPL have not explored the basis of the effect.

Studies by Ganguly *et al.* (2011), Qiao *et al.* (2008) suggest that adiponectin increases the expression of LPL in cardiomyocytes (at 5µg/ml), skeletal muscles (at 10µg/ml) while the research by Sanchez-Gurmaches *et al.* (2011) suggests that adiponectin inhibits LPL expression at 2.5µg/ml. These varying results can be a challenge to interpretation but as the conditions for the various experiments varies, it must be stated that the conditions closest to the ones used in the present study are the ones in the study by Gurmaches *et al* in which 2.5µg/ml (closer to the 3.6µg/ml in the present study) of adiponectin elicited an inhibition in LPL. This may suggest that adiponectin reduces the risk of atherosclerosis by reducing LPL gene expression. Due to the variation in the results, more studies need to be done to further understand the reason for all the variations in the results. The result in figure 3.2.2 suggests that 3.6µg/ml of adiponectin causes an inhibition in the expression of LPA. There are very few studies done previously to explore the effect of adiponectin on LPA gene

expression in endothelial cells. The present study therefore provides further evidence to support the suggestion that adiponectin is vasculo-protective.

In the present study, the basis of the effect of adiponectin on atherosclerotic genes was explored by considering the possibility of the effects of adiponectin seen being mediated via AMPK, ERK, p38 MAPK. Figure 3.2.3- 3.2.5 provides evidence of the involvement of ERK and p38 MAPK and not AMPK.

It is interesting to note that the effect of adipokines in combination are not completely understood and have not been explored by most of the previous studies. In the present study, it was therefore important to explore the effect of a combination of leptin and adiponectin on atherosclerotic and angiogenic genes and also to explore the basis of the effects seen in the combination (by exploring the roles of ERK, AMPK and p38 MAPK). From the results presented in figures 3.1.1- 3.2.11, adiponectin at high (3.6µg/ml) was able to inhibit the up-regulatory effect of leptin on angiogenic and atherosclerotic genes while at low concentrations of adiponectin, the counter-regulatory effect was not as distinct and in some of the real-time PCR results, the combination of HL with LA acted synergistically to increase the expression of the angiogenic genes.

The results also explored the roles of ERK, p38MAPK and AMPK in the effects seen in the combinations. ERK and P38 MAPK appeared to have the most consistent effect on blocking the effect of the combination leading to a gene

expression that was several 1-2 folds less than the up-regulatory effect of leptin seen in angiogenic and atherosclerotic genes.

In the present study, it is interesting to see that LPA and LPL are well expressed in endothelial cells as against the suggestions by previous researchers that LPA and LPL are not expressed in endothelial cells. Also it is interesting to see that for the first time, this study has provided extensive evidence that adiponectin is able to reduce the risk of leptin causing angiogenesis and atherosclerosis (and therefore CVD) in endothelial cells, thereby reducing the risk of CVD.

This study has therefore produced new evidence relating to the link between obesity and CVD and suggests that researchers and clinicians may work together to develop an adiponectin based therapy which may reduce the risk of CVD seen when leptin is high in obese and diabetic individuals.

An area for future research will be to further explore other genes that have not been well expressed such as VEGF and ICAM which are very critical to angiogenesis and atherosclerosis.

Chapter 4

Regulation of insulin and amylin secretion by Leptin and Adiponectin

4.1 Introduction

Diabetes mellitus is a condition that arises from an absence or reduction in insulin secretion or when there is insulin resistance in the presence or absence of adequate insulin secretion (WHO 1999). The role of obesity in the development of diabetes has been linked with adipokines and in type 2 diabetes, adipokines have been linked with beta cell failure or death which leads to a reduction in the cells mass and insulin production and also the development of resistance to insulin (Dunmore and Brown 2013). On the other hand, amylin has also been linked with the development of diabetes as it is said to aggregate at high concentrations to form amyloid which can be deposited in the pancreas and can destroy beta cells. Amylin is also said to inhibit insulin secretion (Ionescu-Tirgoviste *et al.*, 2010).

Previous studies have suggested that monolayer beta cells do not produce insulin in a way that is physiological enough for experimental investigation of insulin secretion and therefore, some research has been done which has lead to the growing of beta cells into clusters of cells that are morphologically similar to islets of Langerhans (thus called “pseudo-islets”) which have been shown to produce insulin in a more co-ordinated way (for example the exhibition of physiological biphasic response to high glucose) (Persaud *et al.*, 2010).

The basis for beta cell dysfunction is not completely understood and based on the complexity of the processes involved, various biological and genetic pathways such as the influence of the PP-1 gene, have been explored and key to this are the pathways by which insulin is secreted and those which insulin influences. Also contributing is the possible effect of amylin on insulin secretion.

Previous studies have tried to investigate what the effect of leptin and adiponectin has on insulin secretion and the basis of these effects. Leptin has been reported to inhibit insulin secretion in the majority of the studies, while adiponectin has been found to have a variable effect on insulin secretion (Lee *et al.*, 2011; Dunmore and Brown 2013), in addition to reducing insulin resistance (Seufert 2004; Okamoto 2006). The roles of leptin and adiponectin are however still not completely understood. This study aimed to find out what the effect of leptin and adiponectin (individually and in combination) is on insulin secretion and to investigate the molecular basis of this effect in terms of the various signaling factors involved and also the genetic basis of the effect of leptin and adiponectin on insulin secretion. The present study also aimed to investigate the effect of these adipokines on amylin secretion.

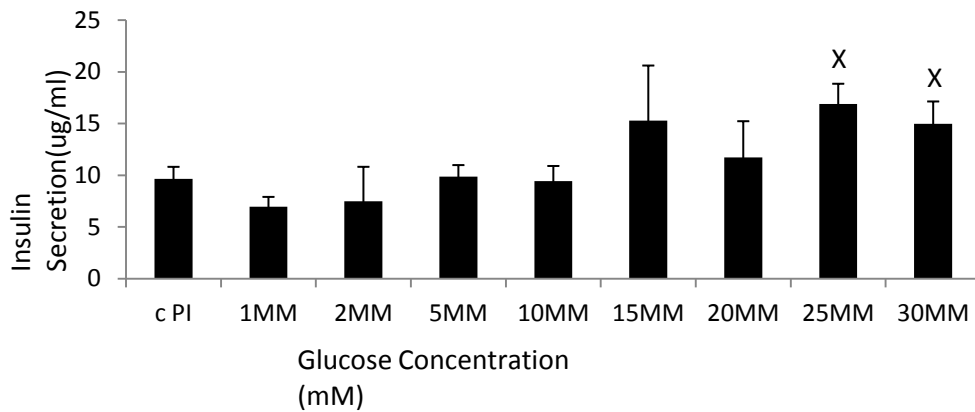
4.2 Materials and Methods

The materials and the methods used for experiments that concern this chapter are detailed in the materials and method chapter (chapter 2)

4.3 Results

4.3.1 Dose response of monolayer β -cells versus pseudoislets to glucose

a)



b)

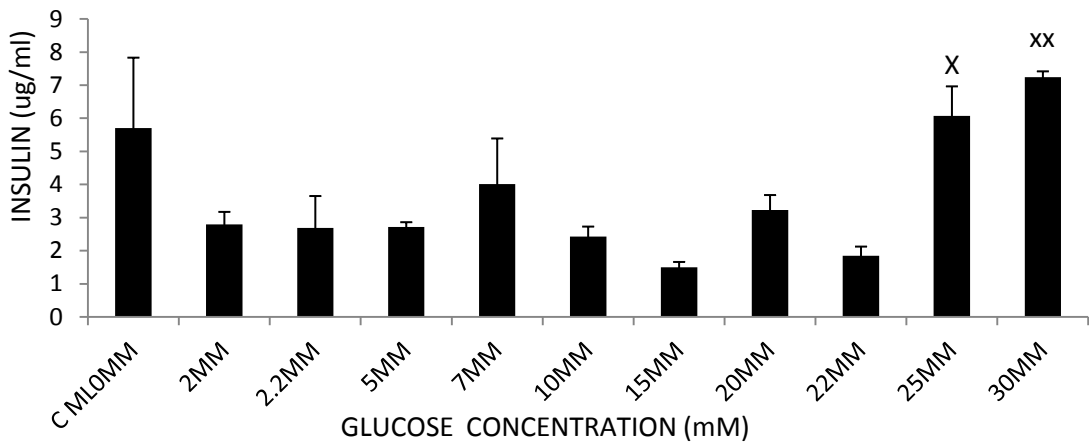
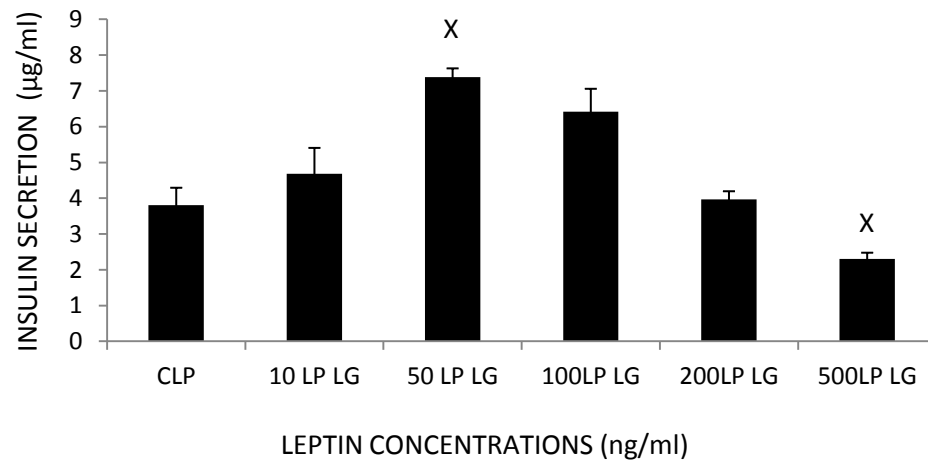


Figure 4.3.1 Dose response of monolayer beta cells compared to pseudoislets. (a) Dose response for pseudoislets and (b) is dose response for monolayer beta cells

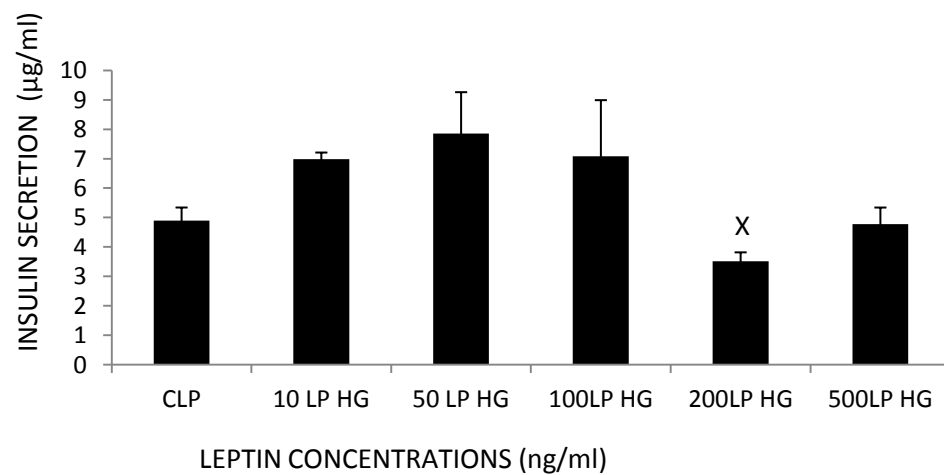
Figure 4.3.1 a and b show that pseudo-islets have better response to glucose than mono-layer cells. There is a significant 3 fold increase in the insulin secretion when insulin produced by 2mM is compared to the 25mM as against a 2 fold increase in monolayers Cells. The pseudo-islets also have better controls and generally produce more insulin than mono-layer beta cells. P-value<0.05 using the students T-Test (2 way) to compare control to sample.

4.3.2 The effect of leptin adiponectin on insulin secretion in low (2.2mM) and high glucose (22mM) in pseudoislets

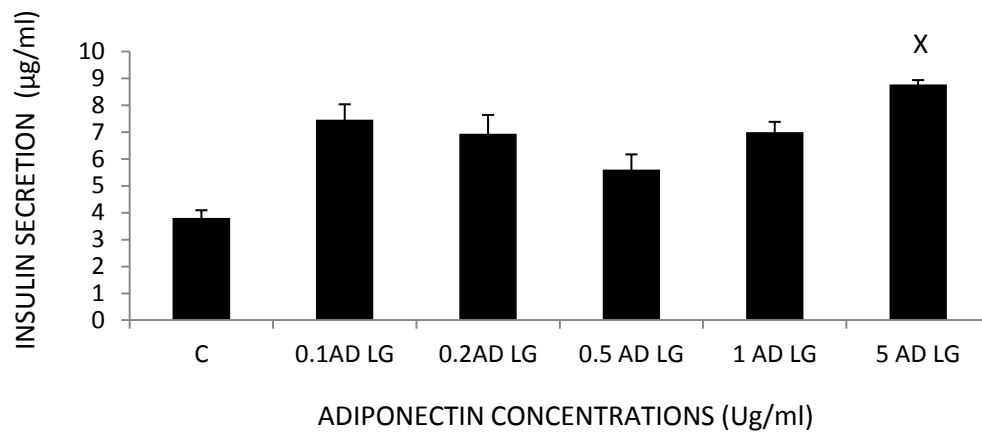
a)



b)



c)



d)

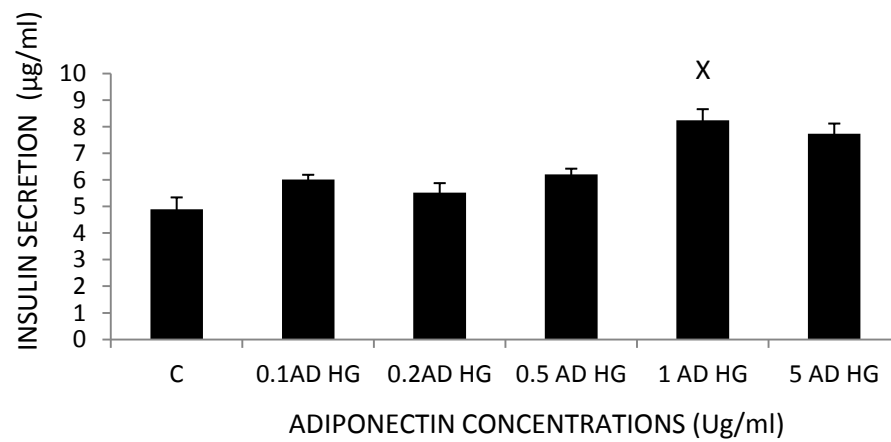
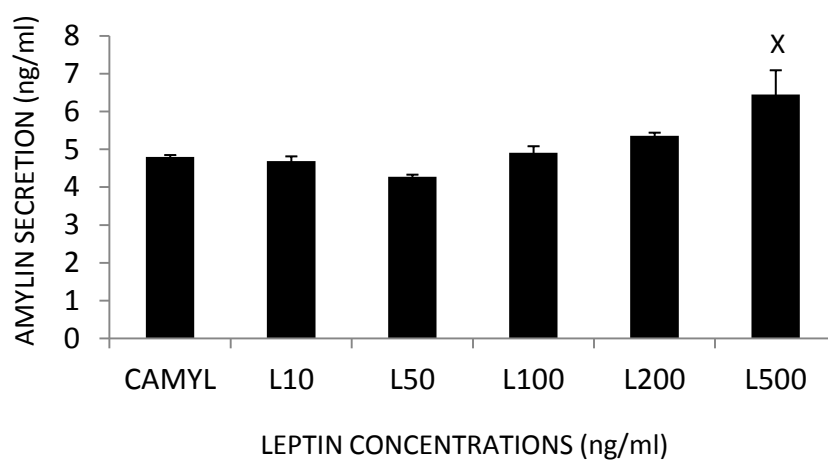


Figure 4.3.2 Effect of leptin and adiponectin in low and high glucose on insulin secretion. a) Effect of leptin in low glucose, b) effect of leptin in high glucose, C) effect of adiponectin in low glucose

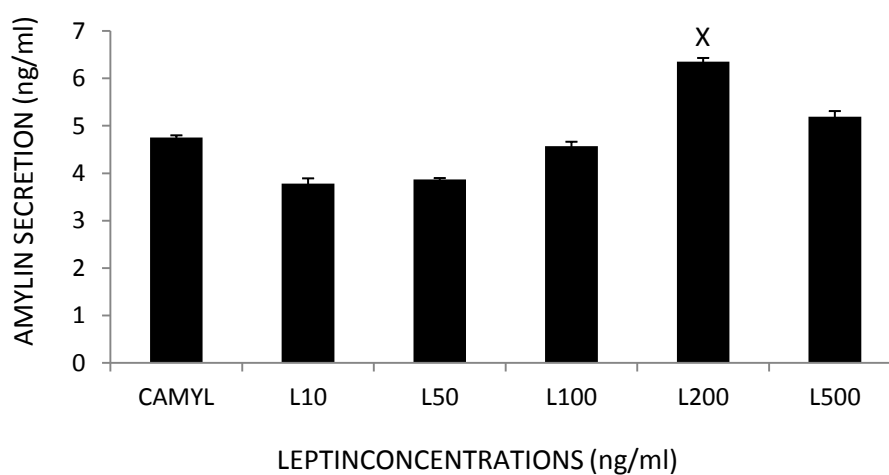
Figure 4.3.2 shows that leptin inhibits insulin secretion and adiponectin increases insulin secretion. A concentration of 200 and 500ng/ml of leptin has a significant inhibitory effect on insulin secretion in high glucose(22mM) and low glucose (2.2mM) respectively , while 5µg/ml and 1µg/ml of adiponectin produce a significant increase in insulin secretion in low high and low glucose respectively. P-value<0.05 using the students T-Test (2 way) to compare control to sample.

4.3.3 Effect of leptin and adiponectin on amylin secretion in low (2.2mM) and high glucose (22mM) in pseudoislets

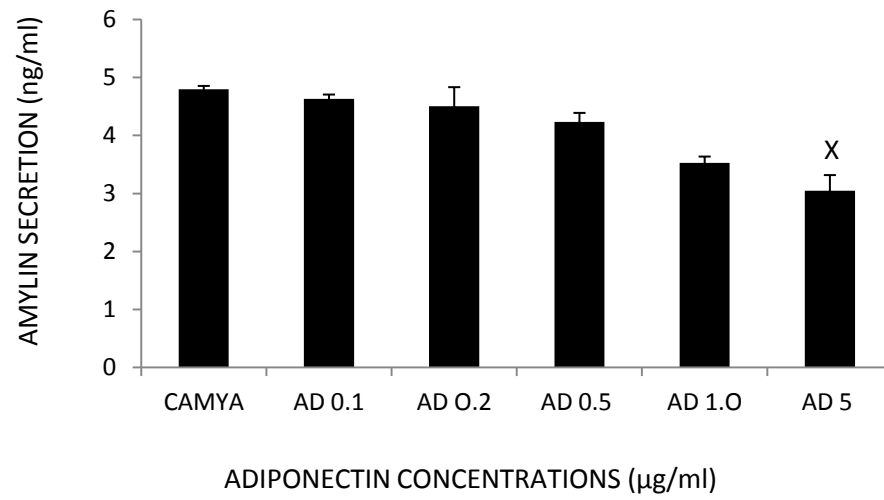
a)



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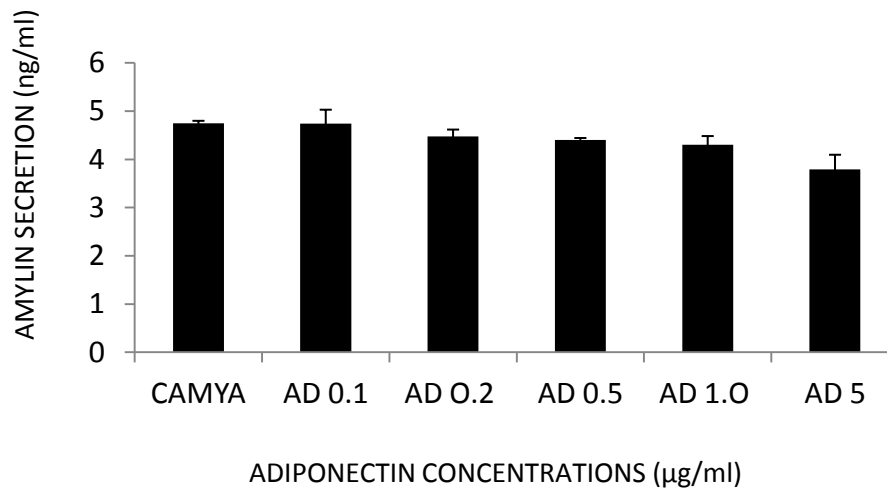
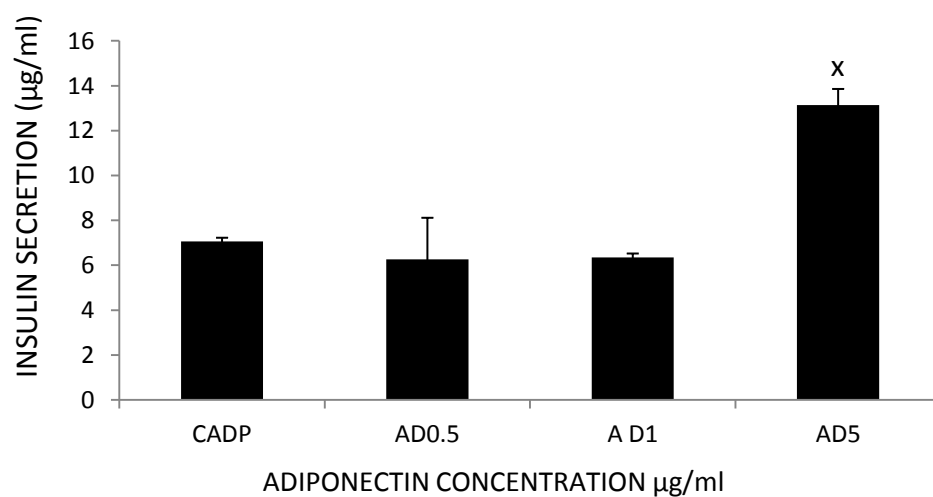


Figure 4.3.3 Effect of leptin and adiponectin in low and high glucose on amylin secretion. a) Effect of leptin in low glucose, b) effect of leptin in high glucose, C) effect of adiponectin in low glucose

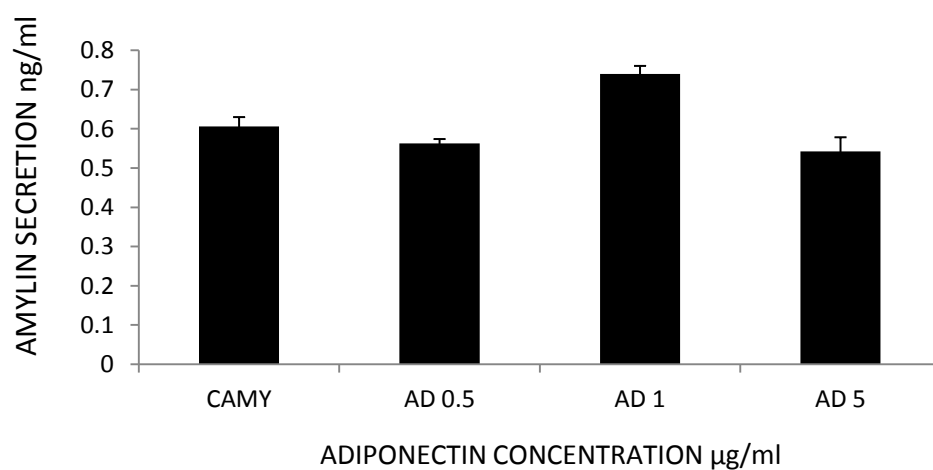
Figure 4.3.3 shows that leptin increases amylin secretion and adiponectin reduces amylin secretion. A concentration of 200 and 500ng/ml of leptin has a significant effect on increasing insulin secretion in high glucose(22mM) and low glucose (2.2mM) respectively, while 5µg/ml of adiponectin produced a significant decrease in amylin secretion in low glucose. P-value<0.05 using the students T-Test (2 way) to compare control to sample.

4.3.4 Comparing the effect of leptin and adiponectin on insulin to the effect on amylin

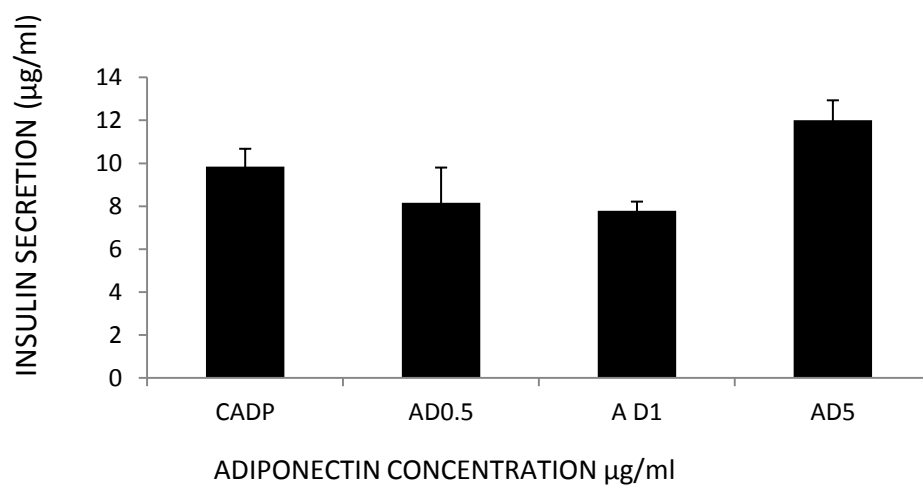
a)



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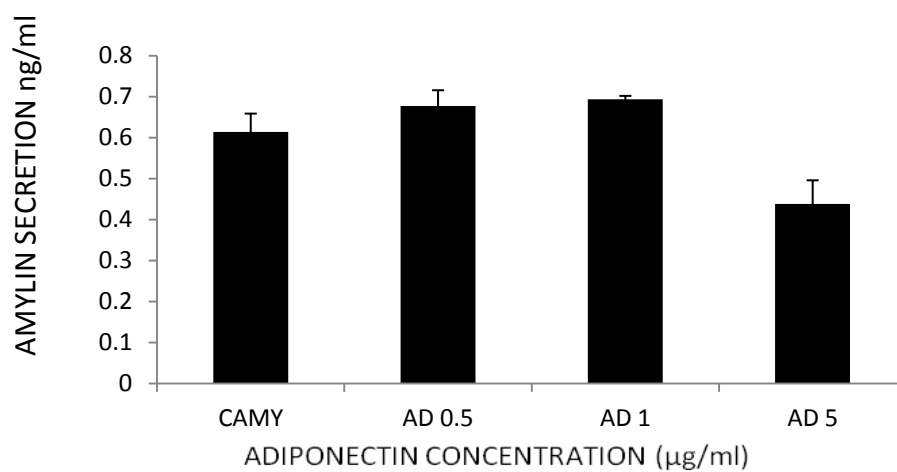
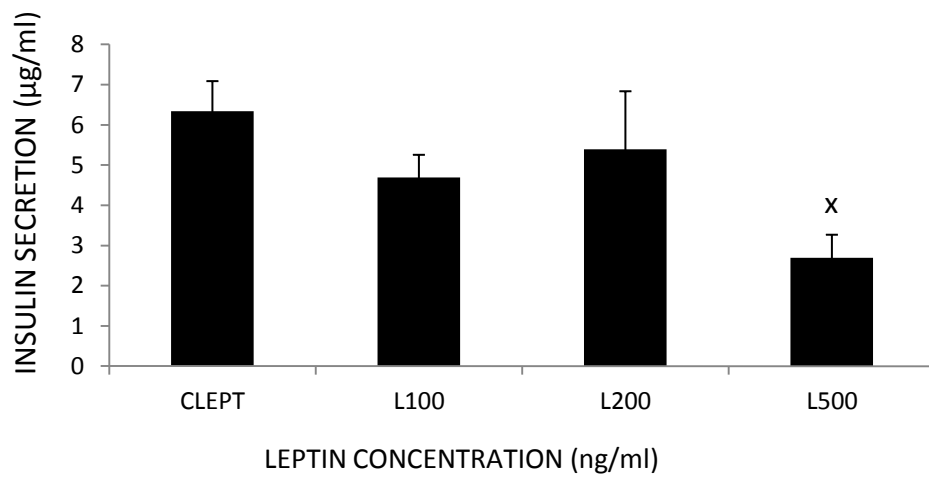


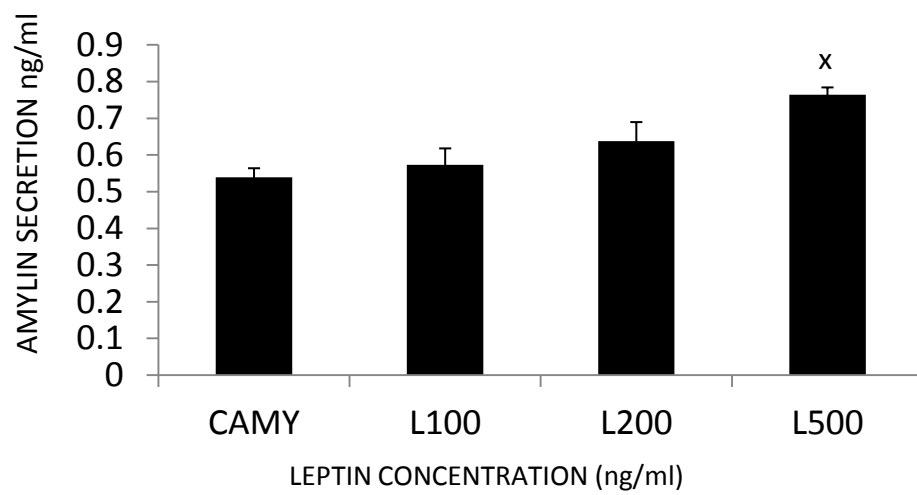
Figure 4.3.4 Effect of adiponectin on insulin compared to amylin in low and high glucose. a)Effect of adiponectin on insulin secretion in low glucose(2.2mM) b)effect of adiponectin on amylin in low glucose (2.2mM), C) effect of adiponectin on insulin secretion in high glucose(22mM), d) effect of adiponectin on amylin secretion in high glucose (22mM)

Figure 4.3.4 shows an increase in amylin secretion which corresponds to a reduction in insulin secretion and vice-versa. The amylin secretion at various concentrations in a treatment where the same cells were assayed for insulin and amylin showed a corresponding inverse pattern of secretion of amylin and insulin in the treatments with adiponectin in low and high glucose. $P\text{-value} < 0.05$ using the students T-Test (2 way) to compare control to sample.

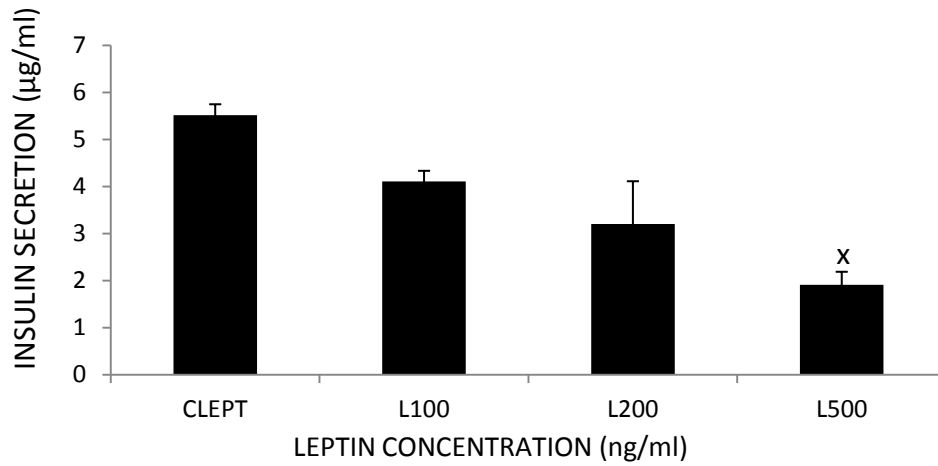
a)



b)



c)



d)

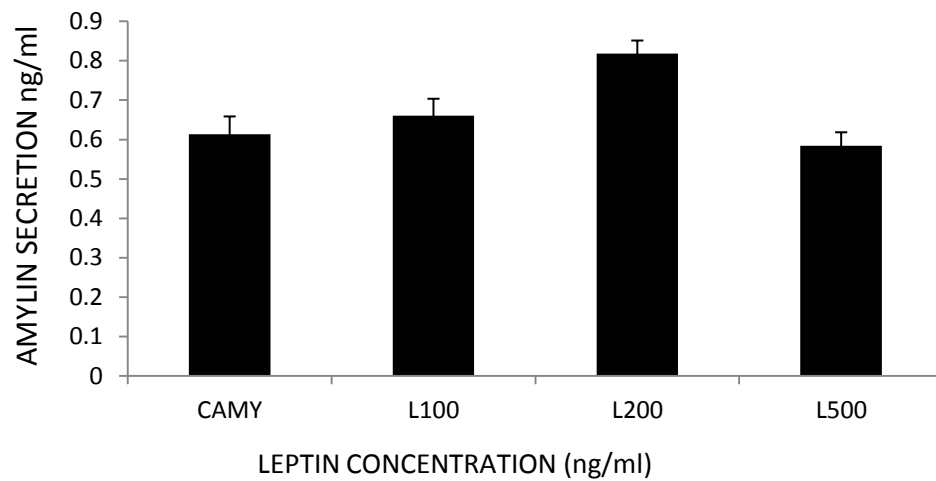
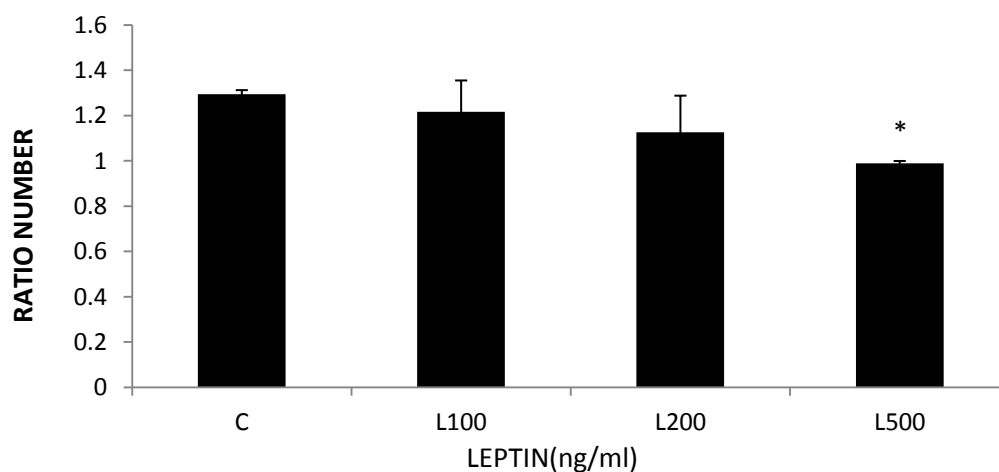


Figure 4.3.5 Effect of leptin on insulin compared to amylin secretion in low and high glucose. a) Effect of leptin on insulin secretion in low glucose (2.2mM) b) effect of leptin on amylin in low glucose (2.2mM), C) effect of leptin on insulin secretion in high glucose (22mM), d) effect of leptin on amylin secretion in high glucose (22mM)

Figure 4.3.5 shows an increase in amylin secretion that corresponds to a reduction in insulin secretion and vice-versa. The amylin secretion at various concentrations in a treatment where the same cells treatments were assayed for insulin and amylin showed a corresponding inverse pattern of secretion of amylin and insulin in the treatments with leptin in low and high glucose. P-value<0.05 using the students T-Test (2 way) to compare control to sample.

4.3.5 Role of Protein Phosphatase 1 (PP-1) in the effect of leptin in insulin secretion

a)



b)

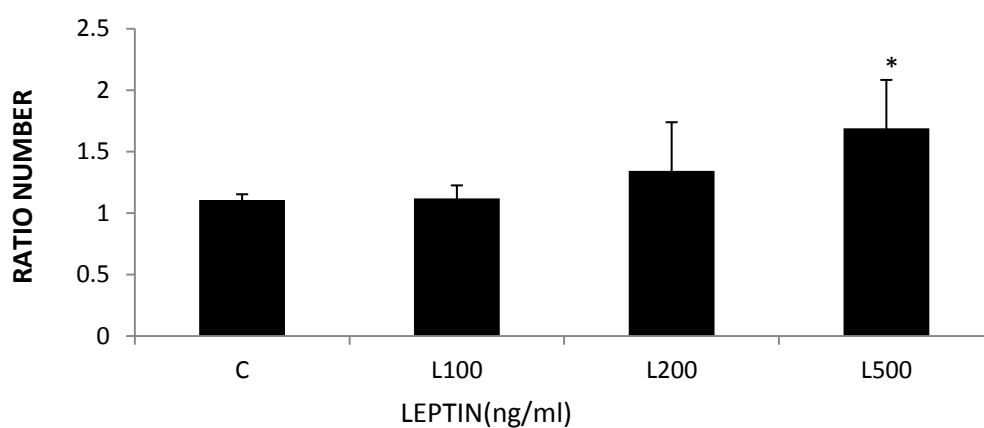


Figure 4.3.6 Effect of leptin on PP-1 gene expression in low and high glucose concentrations (a) effect in low glucose (2.2mM), (b) effect in high glucose (22mM)

Figure 4.3.6 shows that leptin Inhibits PP-1 gene expression. Results show 500ng/ml of leptin in low glucose inhibiting the PP-1 gene expression significantly ($p < 0.05$). P-value < 0.05 using the students T-Test (2 way) to compare control to sample.

4.4.1 Discussion

Type 2 diabetes mellitus is a condition which results from insulin deficiency (either due to inadequate production of insulin or due to resistance to normal amounts of insulin, or both as seen in obese individuals) (WHO1999). Diabetes is strongly linked to obesity and CVD (Ley *et al.*, 2009; Mattu and Randeva 2012). Obesity is a major factor in the development of type 2 diabetes partly because fat cells produce adipokines (such as leptin and adiponectin) and adipokines have been shown by numerous studies to be potential links between obesity and diabetes. Furthermore, obesity has been shown to cause insulin resistance due to the fact that fatty acid together with acyl-CoAs, ceramides, and diacylglycerol may act on the insulin signalling pathway by activating protein kinases such as Protein Kinase C (PKC), Jun kinase (JNK), and the inhibitor of nuclear factor- κ B (NF- κ B) kinase- β (IKK β). These kinases are reported to impair insulin signalling by increasing the inhibitory serine phosphorylation of insulin receptor substrates (IRS) which is the main mediator of insulin signalling thereby inhibiting insulin secretion (Qatanani and Lazar 2007).

It was shown in the study by Maedler *et al* (2008) that leptin which is usually high in obese individuals, inhibits insulin secretion and also causes beta cell death (therefore suggesting that leptin is one of the factors that link obesity to T2DM), while adiponectin increases insulin sensitivity and insulin secretion (Seufert 2004). Insulin secretion has been used extensively as an experimental

parameter to assess beta cell function (Persaud 2010). In order to assess what the possible basis of diabetes is, it was considered important in the present study to grow beta cells as pseudo-islets (which have not been widely used but have been shown by researchers to produce more insulin and secrete it in a more physiological manner than monolayer beta cells) and also to explore the effect of leptin and adiponectin on amylin secretion (Persaud 2010). .

Beta cells were grown in 24 well plates coated with gelatin leading to formation of pseudoislets and then treated in the presence of Krebs Hepes buffer with low and high glucose (2.2mM and 22mM respectively) together with a range of adipokine concentrations. The insulin and amylin secreted by the cells were then assayed. Results from this study suggest that leptin predisposes to T2DM (due to a reduction in insulin secretion and increase in amylin secretion) while adiponectin reduces the risk of T2DM (increases insulin secretion and inhibits amylin secretion. Amylin secretion and insulin secretion were compared and the insulin/amylin ratio in most of the samples was high compared to the reported normal ratio.

Before initiating the adipokine experiments, it was important to find out if the cells responded to glucose with insulin release therefore the beta cells were all treated with a range of glucose concentrations in the absence of adipokines. In this study pseudoislets exhibited a dose response of increasing insulin secretion over a range of glucose concentrations, although this was only

significant at the higher glucose concentrations. In monolayer cells the response was relatively flat until higher concentrations of 25-30mM glucose which caused a 1.5-3 fold increase ($p < 0.05$) in insulin secretion when compared to the control or 1mM and 2mM treatment. (Fig 4.3.1), These results further support the findings in the study by Chowdhury *et al* (2013) which suggested that there is more insulin secretion in pseudoislet compared to monolayers Cells. The data in figure 4.3.1 also suggest that pseudoislets have a better control response (at zero glucose concentration) when compared with monolayer cells.

The results in figure 4.3.2 show a significant inhibition ($p < 0.05$) of insulin secretion by 200ng/ml (12nM) leptin in high glucose (22mM) and 500ng/ml (30nM) in low glucose (2.2mM). In the previous studies (Lee *et al.*, 2011, Brown *et al.*, 2002, Roduit and Thorens 1999, Zhao *et al.*, 1998) beta cells have been treated with leptin concentrations varying from 0.1 to 100nM and in various concentrations of glucose (representing very low glucose and high glucose as can be seen in diabetics.) The results of the current study provide further evidence which confirms the suggestion from previous research that leptin inhibits insulin secretion. In the present study, there is evidence to suggest that the pattern seen in inhibition might be linear as suggested by Brown *et al.* (2002) or an inverted “U” shape as suggested by Roduit and Thorens (1999) where 10nM and 100nM of leptin was shown to have a significant inhibitory effect on insulin secretion. This is in contrast to the “U” shape reported by Brown *et al.* (2002) and the reason for this may be as a result of the batch of

cells used. The implication of this may be the possibility of obesity (high leptin) in diabetes leading to a further inhibition in insulin secretion which complicates the insulin resistance that may be present in obese diabetics as suggested by Zhao *et al.* (2004).

Adiponectin at 1 and 5 µg/ml increased insulin secretion significantly ($p < 0.05$) in the present study. This may suggest the possibility of adiponectin increasing insulin secretion even under diabetic conditions (very low and very high glucose), and this provides further evidence supporting the findings by Okamoto *et al.* (2008).

The study of the effect of adiponectin on insulin secretion has been controversial as there has been varying views on the effect of adiponectin in low and high glucose on insulin secretion. Previous studies by Gu *et al.* (2006) suggest that 0.1 µg/ml (2.5 nM) of adiponectin causes an increase in insulin secretion only in high glucose (16.7 mM) while Okamoto *et al.* 2008 shows that 10 µg/ml (300 nM) of adiponectin caused an increase in insulin secretion only in low glucose (5.6 mM). In the present study 0.1, 0.2, 0.5, 1, and 5 µg/ml of adiponectin were used in the treatment of beta cells in low (2.2 mM) and high glucose (22 mM) and the result are shown in figure 4.3.3. These variations in results may be a consequence of the differing concentrations of adiponectin and glucose used in these experiments and may therefore require standardization on future studies.

In investigating the effect of leptin and adiponectin on amylin secretion, 0.1, 0.2, 0.5, 1 and 5µg/ml (3, 6, 15, 30 and 150 nM) of adiponectin and 10, 50, 100, 200 and 500ng/ml (1, 3, 6, 12, 30nM) of leptin was used to treat beta cells. In the *in-vivo* study in humans by Hwang *et al.* (2008) it was suggested that leptin had no effect on amylin secretion, while the *in vitro* study in rodents by Karlsson *et al.* (1998) suggested that long term treatment with leptin (1-100nM for 2-5 days) inhibits amylin secretion at various glucose concentrations from 1.7mM to 16.7mM. In the present study, the result depicted in figures 4.3.3 to 4.3.5 gives a different perspective as it suggests that leptin increases amylin secretion significantly at 500ng/ml (30nM). The difference seen in these studies may be as a result of the use of different species of subjects, the use of pseudoislets in the present study as against the treatment of pancreatic islets in previous studies and also the varying periods of treatment with leptin. This suggests that more studies may need to be done under similar conditions in the future to make comparison easier. As seen in the effect of leptin on amylin secretion (with very few studies done prior to the present study), there was no previous directly comparable study done previously on the effect of adiponectin on amylin secretion. Figures 4.3.3 to 4.3.5 in the present study show that adiponectin inhibits amylin secretion significantly at 5µg/ml (150nM) in low and high glucose concentrations. The results from the present study may be tentatively taken to suggest that leptin may predispose to diabetes as a result of an increase in amylin secretion leading to an increase in amyloid accumulation,

while adiponectin may reduce the risk of diabetes as a result of a decrease in amylin secretion.

Amylin is a peptide that is co-secreted with insulin from the pancreas and one of the factors that keeps the glucose concentration in the blood stable by delaying gastric emptying and intestinal absorption when there is an increase in the amount of glucose in the body and vice-versa when there is a hypoglycaemia (Karlsson 2000). In the current study, pseudo-islets were treated with adipokines and the assay of the insulin and amylin secretion for the same sample was done to explore the hypothesis that the amylin/insulin ratio may change insulin secretion.

In figure 4.3.4, there appears to be an inverse correlation between insulin and amylin secretion in the samples treated with 1µg/ml and 5µg/ml of adiponectin. The amylin secretion in the sample treated with 1µg/ml is increased relative to the control and this is contrasted with a relative reduction in insulin secretion in the same treatment, and vice-versa in the treatment with 5µg/ml where the amylin secretion is relatively reduced when compared to the control and this is combined with a significant increase in insulin secretion. This is in agreement with the research by Karlsson *et al.*, (1998) and is in contradiction to the suggestions by Tedstone *et al.*, (1990) which suggested that amylin had no effect on insulin secretion. A similar pattern is seen in the cells treated in high glucose as the u shaped pattern of increase in insulin secretion by adiponectin correlates with the inverted “u” shape of the amylin secretion. This suggests an equal and opposite action on amylin compared to insulin secretion. This

suggest that high adiponectin, in low or high glucose even with an increase in amylin secretion may be able to boost insulin secretion.

In figure 4.3.4, the pattern of the increase in amylin correlates with a corresponding reduction in insulin secretion in cells treated with leptin in low glucose. There is an exact equal and opposite significant reduction in insulin secretion which contrasts with an increase in amylin secretion. In the cells treated with leptin in high glucose (figure 4.3.5), there is a corresponding reduction in insulin secretion which correlates with a pattern of increase in insulin secretion in the treatments with 100, and 200ng/ml, and unlike this, the treatments with 500ng/ml of leptin in high glucose appears to inhibit both insulin and amylin secretion which suggest that at very high leptin concentrations, there may be a decline in all beta cells functions. This is in agreement the suggestions by Tedstone *et al.*, (1990). The normal ratio of insulin to amylin is usually 100:1.

The results show a consistent significant inhibition of insulin secretion by 500ng/ml leptin and based on the leptin pathways explored, it has been suggested by Kuehnen *et al.*, (2011) that Protein phosphatase (PP-1) mRNA and protein expression is down-regulated by leptin, which leads to the reduction of PP-1 enzyme activity in beta cells. In addition, glucose-induced insulin secretion was said to be inhibited by nuclear inhibitor of PP-1, which was in part mediated by a reduction of PP-1-dependent calcium influx into beta cells. Therefore in this study, beta cells treated with various concentrations of leptin (100, 200 and 500ng/ml) in low (2.2mM) and high (22mM) glucose were

collected, mRNA extracted from them and reverse transcription PCR done to assess the effect of leptin on the expression of PP1A gene. Figure 4.3.6 shows that 500ng/ml of leptin had a significant inhibitory effect on the expression of PP1A gene in low glucose which is in agreement with the findings in the study by Kushner *et al.*, (2011) which suggests that the inhibition of PP1A gene which leads to a reduction in calcium influx and the secretion of insulin, is one of the pathways by which leptin inhibits insulin secretion and therefore predisposes to diabetes mellitus.

From the results of the current study, it may be suggested that clinicians, researchers and pharmacologists can explore possible pharmacotherapeutic means of boosting adiponectin levels *in-vivo* and ways of reducing the concentrations of leptin and regulating the concentration of amylin as adiponectin had be shown to boost insulin secretion while leptin and amylin appear to inhibit insulin secretion making the T2DM condition worse.

It is interesting to note that leptin and adiponectin have opposite effects on insulin compared with amylin secretion, thereby altering the insulin/amylin ratio with a possibility of this contributing to either making diabetes better or worse in an individual and this balance can be explored by pharmacotherapeutic means to improve diabetes. Also it is interesting to note that adiponectin in the present study increases the insulin secretion as against the contrasting positions of researchers some of who have suggested that adioponectin may not have any

effect on insulin secretion. Also interesting is the results which shows for the first time that adiponectin inhibits amylin secretion.

As a result of the fact that the effect of adipokines on amylin secretion has been poorly studied, the conclusions on the study on amylin secretion should be viewed with caution as there is obviously a strong necessity to do more research on the effect of adipokines in a wider range of species and with various concentrations of adipokines. For future work, it will be interesting to explore the effect of adding amylin analogues to treat beta cells in order to objectively assess the quantities of amylin that has inhibitory effect on beta cells insulin secretion. Furthermore, there appears to be the need to confirm the possible effects of the insulin/amylin ratios in more physiological relevant models such as human islets and *in-vivo*. It will also be useful if more work is done to explore further the reason why monolayer beta cells produce so much insulin when placed in no Glucose.

Chapter 5

Plasma levels of leptin and adiponectin in diabetic patients and relationship to beta cell failure and cardiovascular disease

5.1 Introduction

Type 2 diabetes mellitus is a condition that occurs as a result of insulin deficiency (either due to inadequate production of insulin or the inability of insulin to carry out its normal function in target tissues) (WHO 1999).

Data collected by the WHO shows that world-wide, diabetes affects about 520 million people (WHO 2014). More recently, obesity has been linked with T2DM and CVD. Research has narrowed down on various peptide factors called adipokines as the link between obesity and CVD and T2DM (Bravo *et al.*, 2006, Barnes 2011).

In the present study, the effect of leptin and adiponectin on beta cell function and the risk of CVD have been explored. *In vitro*, leptin has been shown to promote the development of T2DM and CVD while adiponectin has been shown to reduce the risk (Bravo *et al.*, 2006, Barnes 2011).

The concept of using the leptin:adiponectin ratio as an index has been used in research for some time as it is said to be a good predictor of insulin resistance (Rashid 2013) and a good atherogenic index in T2DM (Satoh *et al.*, 2004) and it was suggested that the leptin/adiponectin ratio is a better atherogenic index

than either leptin or adiponectin on its own. Ling *et al.* (2014) also proposed that the higher the leptin/adiponectin ratio, the higher the risk of developing insulin resistance.

The G-gap is an empiric measure of disproportion or difference seen between HbA_{1c} and fructosamine which are the two principal methods of estimating glycaemic control indirectly (Nayak *et al.*, 2013). The G-gap has been suggested to be linked with diabetes complications (retinopathy, nephropathy, macrovascular disease, and mortality). In the present study, these complications were explored in diabetic patients and potential associations between leptin/adiponectin ratio, the G-gap and clinical outcomes were explored. The G-gap is used in this study in a similar way to the study by Nayak *et al.* (2013) and is proposed as a measure of the deviation of glycated HbA_{1c} away from its expected value, such that a negative G-gap is taken as meaning a lesser level of glycation than expected and a positive G-gap meaning a higher level of glycation of proteins. Increased glycation of proteins has been linked with hyperglycemia in diabetes mellitus and is a strong indicator of possibility of complications in diabetes. G-gap is calculated using the following formula;

$$\text{G-gap} = \text{True HbA}_{1c} - \text{FHbA}_{1c} \text{ (Fructosamine-derived standardized predicted)}$$

$$\text{FHbA}_{1c} = \{[(\text{fructosamine} - \text{mean fructosamine}) / \text{SD fructosamine}] \times \text{SD HbA}_{1c}\} + \text{mean HbA}_{1c}$$

(Nayak *et al.*, 2013)

In the clinical part of the study, we examined possible associations of the leptin/adiponectin ratio with the complications seen in patients with T2DM and the relation of this to the G-gap.

5.2 Materials and Methods

The materials and the methods used for experiments that concern this chapter are detailed in the materials and method chapter (Chapter 2)

A summary of how the clinical part of the research was carried out is as reported as follows. The data collected in the present study required the recruitment of patients from the cohort of those with diabetes treated at the Diabetes Centre, New Cross Hospital, Wolverhampton, and approval of the relevant local U.K. National Health Service Research Ethical Committee was sought for this. Routine blood samples had been collected for the analysis of all HbA1c and fructosamine estimations at New Cross Hospital in Wolverhampton. The result of this analysis were then used for the identification and selection of all adults with diabetes (≥ 18 years of age) who had paired estimations of HbA1c and fructosamine performed on the same day from the same sample set and after this, clinical information was taken from the diabetes registry and linked to this dataset.

As this part of the clinical part of the study was done in collaboration with Nayak et al, many of the conditions used in this part of the study are similar to those used by Nayak *et al.* (2013). In accordance with the suggestions by Nayak *et al.*, the diabetes register, which is validated to be 99% accurate for the

identification of known diabetes and for mortality status in linkage with the National Health Service Strategic Tracing Service was used. Patients that were excluded from this study included pregnant women, those with creatinine levels greater than 200 $\mu\text{mol/L}$, those with a known hemoglobinopathy and those with an abnormal electrophoretic pattern on HbA1c testing. The G-Gap was then calculated for all the selected patients. Those with the clearest consistently negative or positive G-Gap was invited to participate in the study and 150 patients participated in the study having given fully informed consent. Patients were invited to attend the clinic where fasting blood samples were collected in heparinised tubes. Blood samples were centrifuged to separate blood cells and plasma and the cells used in another part of the G-Gap project. Plasma samples were frozen at -80°C until used for assays. The patient cohort included 75 with a positive G-Gap and 75 with a negative G-Gap.

The samples collected were then processed and analysed for the leptin and adiponectin concentrations and this was correlated with the G-gap (see formula above) and these parameters linked with complications in diabetics and also the levels of complications seen in the patients. The correlation was done using the minitab software and correlation grouped as strong (0.75-1.00), moderate (0.5-0.75) and < 0.5 is a weak correlation. P values are < 0.05 or <0.00 to be significant.

5.3 Results

5.3.1 Plot of Leptin/Adiponectin ratio for GGAP positive and negative

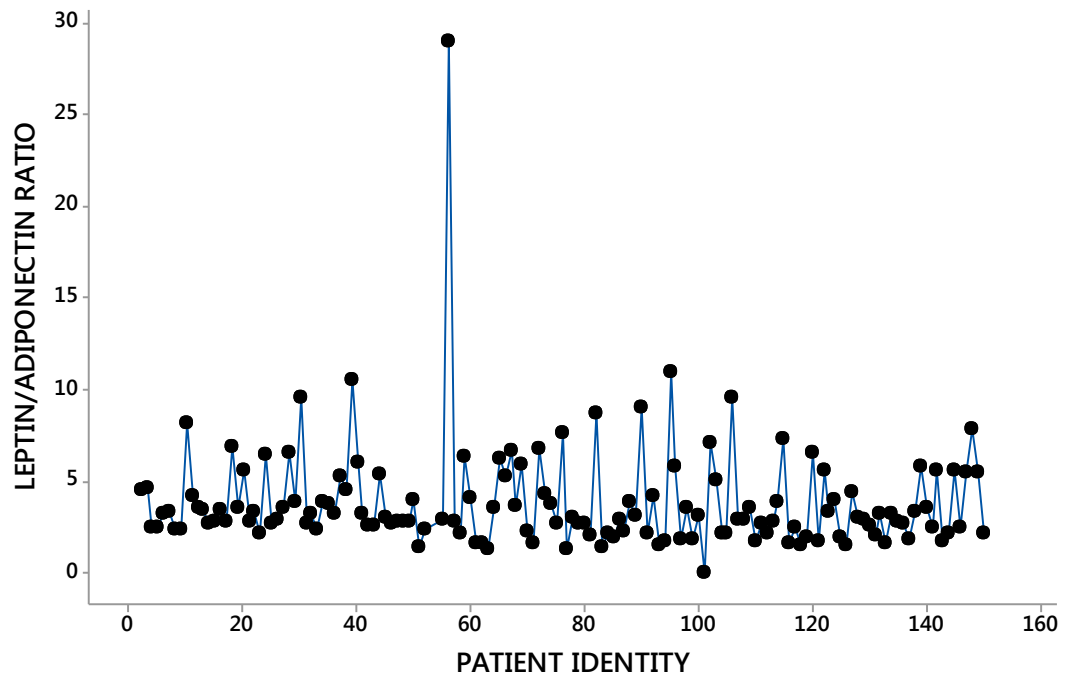
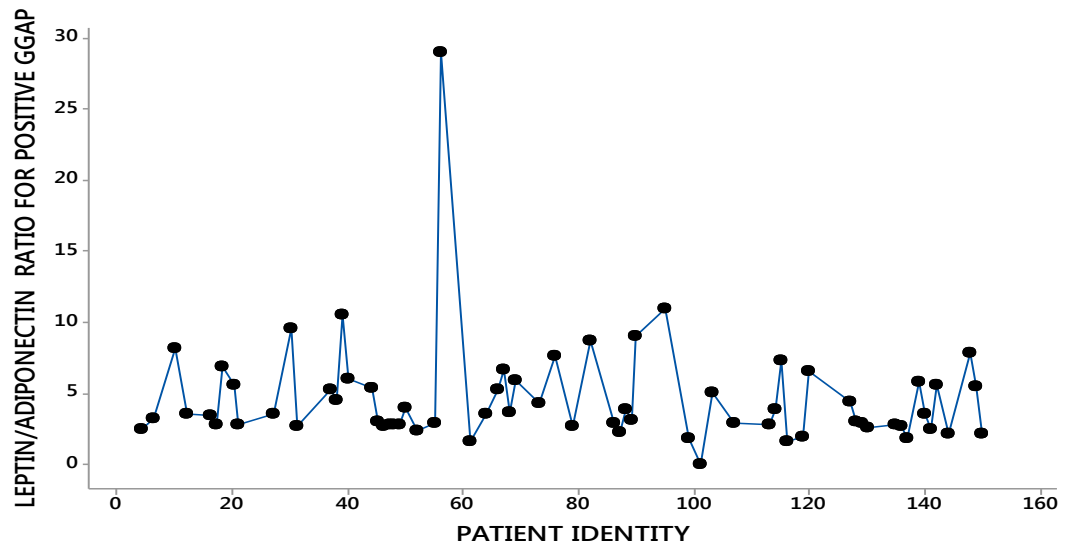


Figure 5.1 Scatterplot of leptin/adiponectin ratio for all samples.

Figure 5.1 shows the leptin/adiponectin ratio is plotted as a scatterplot in order to show the levels of the L/A ratio in all the patients (sample ID 2-150). This shows that most of the samples have L/A ratio less than 15 with majority being below 5.

5.3.2 Distribution of patients with positive and negative G gap

a)



b)

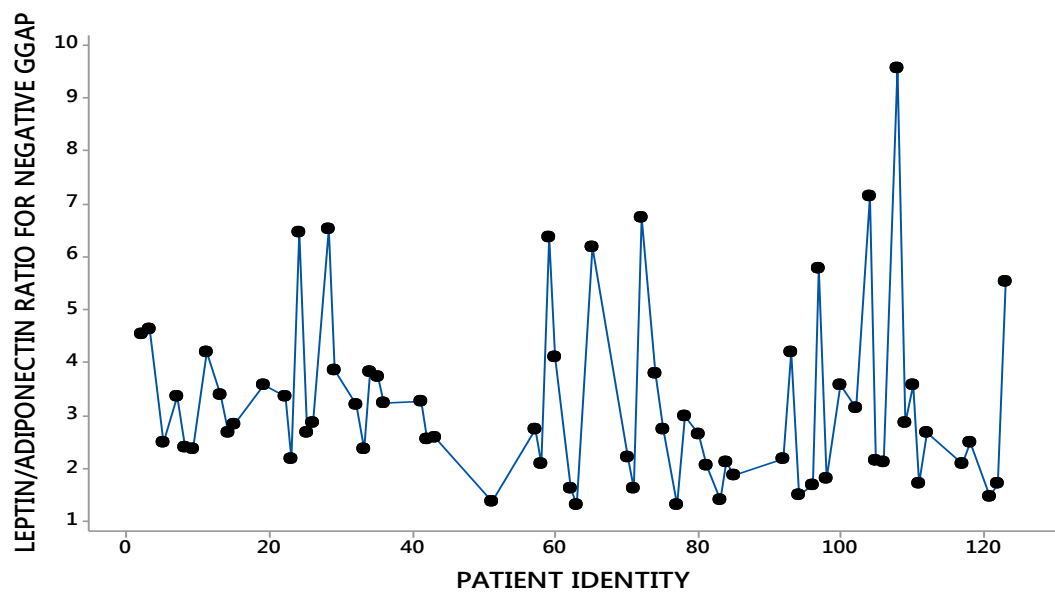
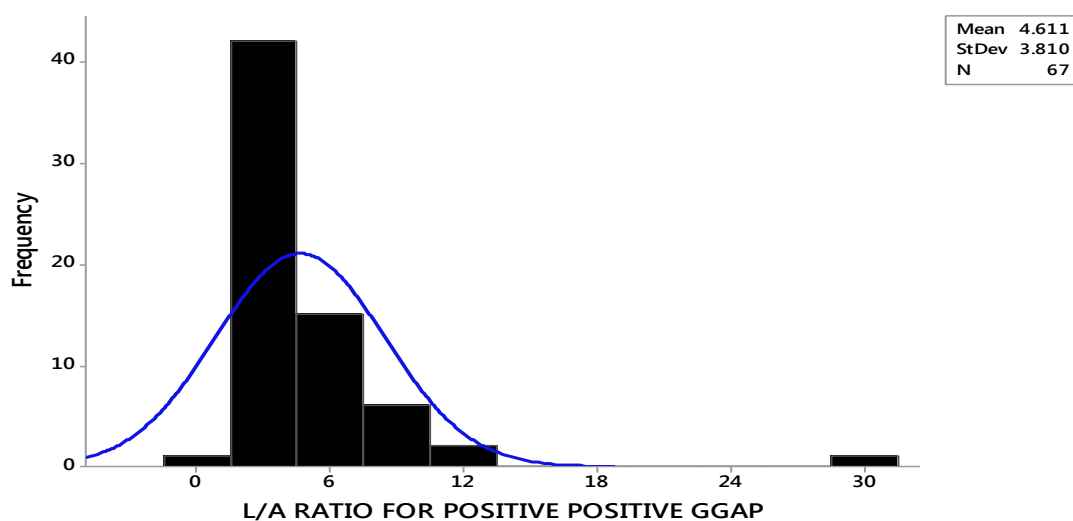


Figure 5.2 Scatterplot of the distribution of distribution of patients with positive and negative G-Gaps. (a) Distribution of patients with positive G-gap, b) distribution of patients with negative G-Gaps

Figure 5.2 shows the scatterplot for the distribution of patients with positive and negative G-gaps. It shows that the separate distribution of L/A ratio in positive has a higher range (Majority being from 2.5-10) than the lower L/A ratio in the negative (mostly 1.5-6.5).

5.3.3 Frequency distribution of L/A ratio in Positive vs. Negative GGAP

a)



b)

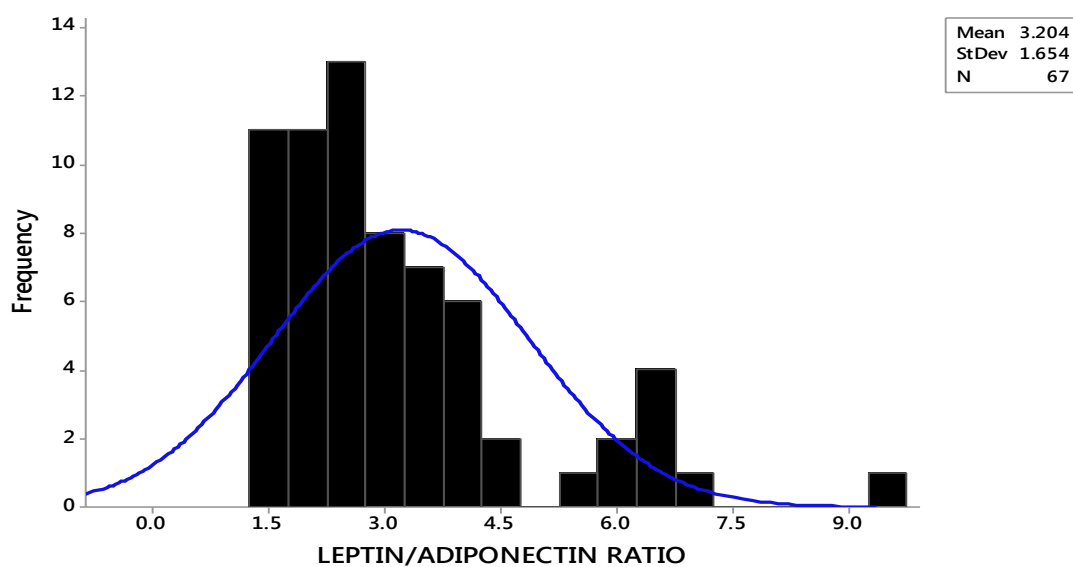


Figure 5.3 Frequency of the distribution of patients with positive and negative G-Gaps. a) Frequency of patients with positive G-Gap, b) Frequency of patients with negative G-gap

Figure 5.3 shows the histogram of the frequency distribution of L/A ratio for patients with negative and positive GGAP. (a) Shows 43 of the 67 patients with GGAP negative being below the average value of 3.204, while there are 41 of the patients with a positive GGAP being below the average for positive GGAP (4.611)

5.3.4 Correlation of Leptin/Adiponectin versus leptin and adiponectin alone with Predictors of complications

PARAMETERS	LAR	
	R	p-value
BMI	0.752	0.000 (<0.001)
HDL	-0.304	0.000
eGFR	-0.080	0.336
GGAP	0.356	0.000
CHOLESTEROL	-0.005	0.958
ADIPONECTIN	-0.057	0.494
LEPTIN	0.779	0.000
CVD RISK	0.109	0.191
uACR	-0.008	0.921
ALBUMIN	-0.306	0.000
RETINOPATHY	0.101	0.228

Table 5.1 Correlation of LAR with risk parameters in diabetics

PARAMETERS	LEPTIN	
	R	p-value
BMI	0.473	0.000 (<0.001)
HDL	-0.165	0.048
eGFR	-0.189	0.023
GGAP	0.266	0.001
CHOLESTEROL	0.022	0.798
ADIPONECTIN	0.166	0.046
LEPTIN	-	-
CVD RISK	0.070	0.401
uACR	0.070	0.401
ALBUMIN	-0.287	0.000
RETINOPATHY	-0.015	0.854

Table 5.2 Correlation of Leptin levels with risk parameters in diabetics

PARAMETERS	ADIPONECTIN	
	R	p-value
BMI	-0.083	0.322
HDL	0.188	0.024
eGFR	-0.055	0.513
GGAP	0.054	0.520
CHOLESTEROL	-0.038	0.664
ADIPONECTIN	-	-
LEPTIN	0.166	0.046
CVD RISK	-0.161	0.048
uACR	-0.095	0.255
ALBUMIN	0.054	0.522
RETINOPATHY	-0.053	0.530

Table 5.3 Correlation of adiponectin levels with risk parameters in diabetics

5.3.5 Distribution of leptin/adiponectin ratio vs. Body Mass Index (BMI)

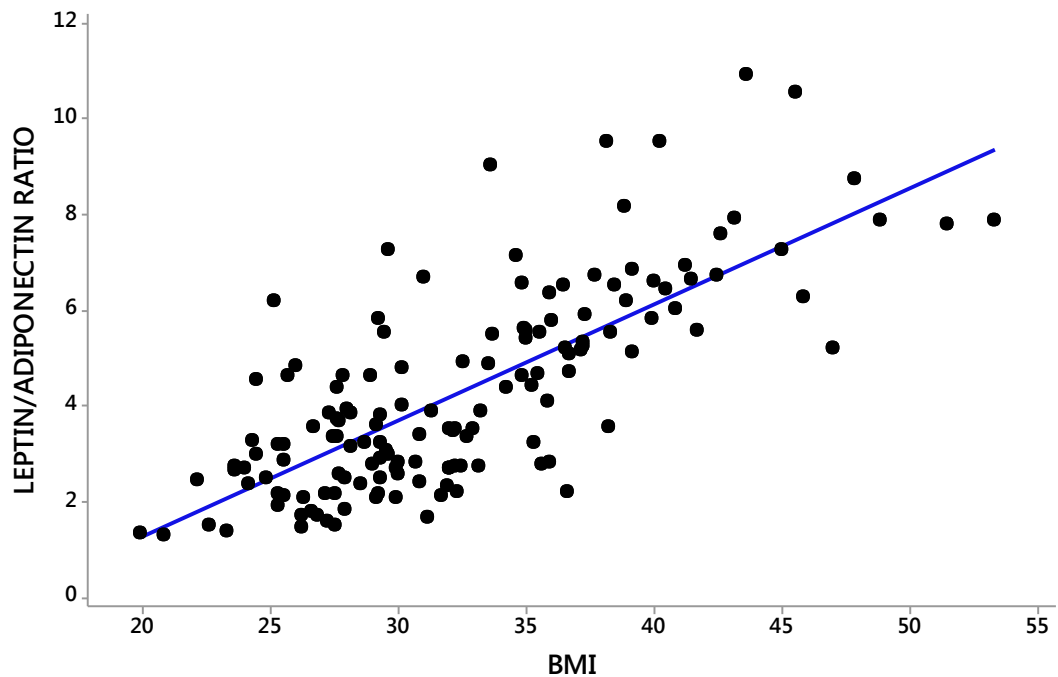


Figure 5.4 Scatterplot of the distribution of Leptin/Adiponectin ratio plotted against Body Mass Index (BMI). This shows a positive linear regression

Figure 5.4 shows that there is a positive linear relationship between L/A ratio and the BMI with a correlation factor of 0.752 ($p < 0.001$) showing a strong correlation between the L/A ratio and the BMI.

5.3.6 Correlation of the Leptin/adiponectin ratio vs. Leptin

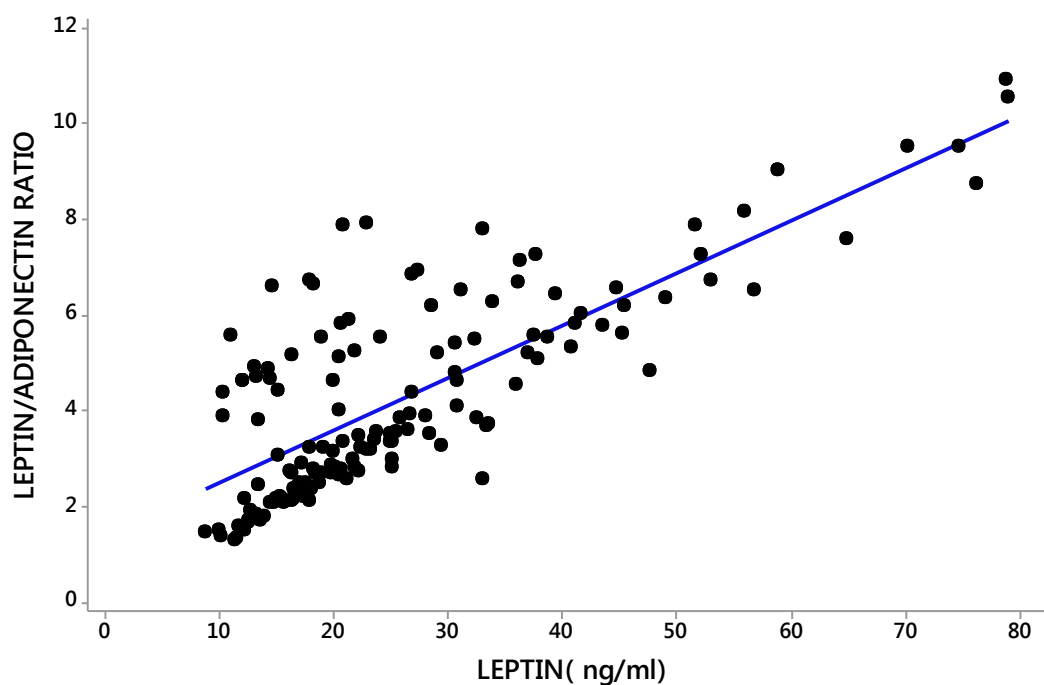


Figure 5.5 Scatter plot of the distribution of leptin/adiponectin ratio plotted against leptin. This shows a positive regression line.

Figure 5.5 shows that there is a linear relationship and a strong correlation ($r=0.779$ and $p<0.001$) between the leptin/adiponectin ratio and the leptin levels in the patients. This shows leptin to be positive linear relationship to L/A and also most of its possible complications.

5.3.7 Correlation of leptin/adiponectin ratio with High density lipoprotein levels (HDL)

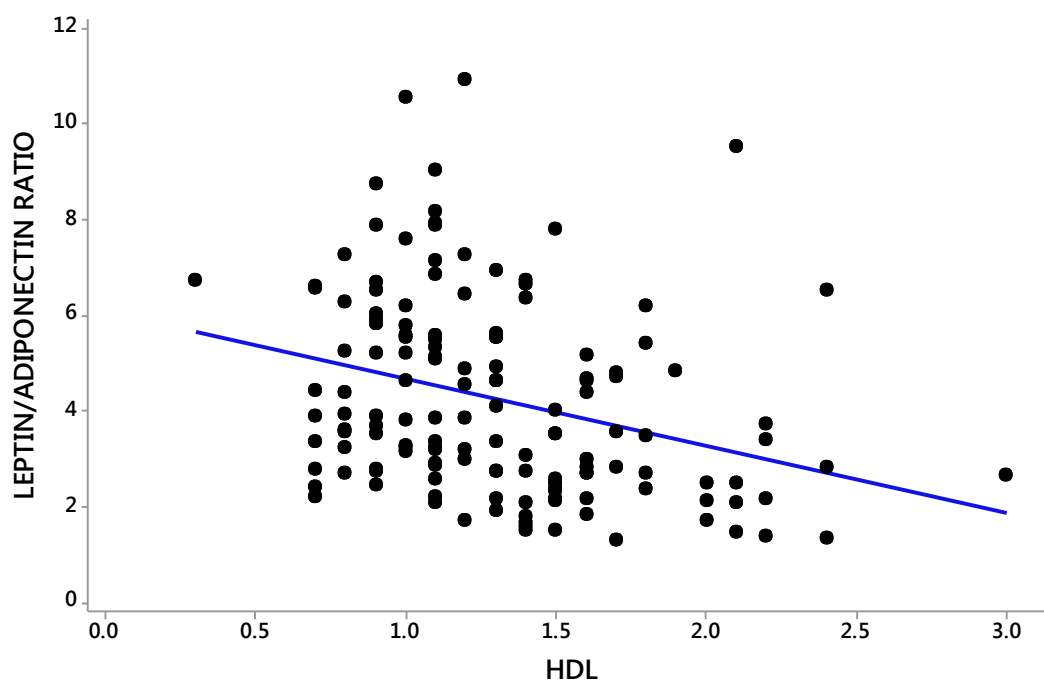


Figure 5.6 Scatterplot of distribution of Leptin/Adiponectin ratio plotted against the levels of High Density Lipoprotein.

This shows a weak negative linear correlation (-0.304 with $p < 0.001$) between the leptin/adiponectin ratio and the concentration of HDL in the patients' blood showing the link between L/A ratio and possible cardiovascular complications.

5.3.8 Correlation of Leptin/adiponectin ratio with the Albumin levels

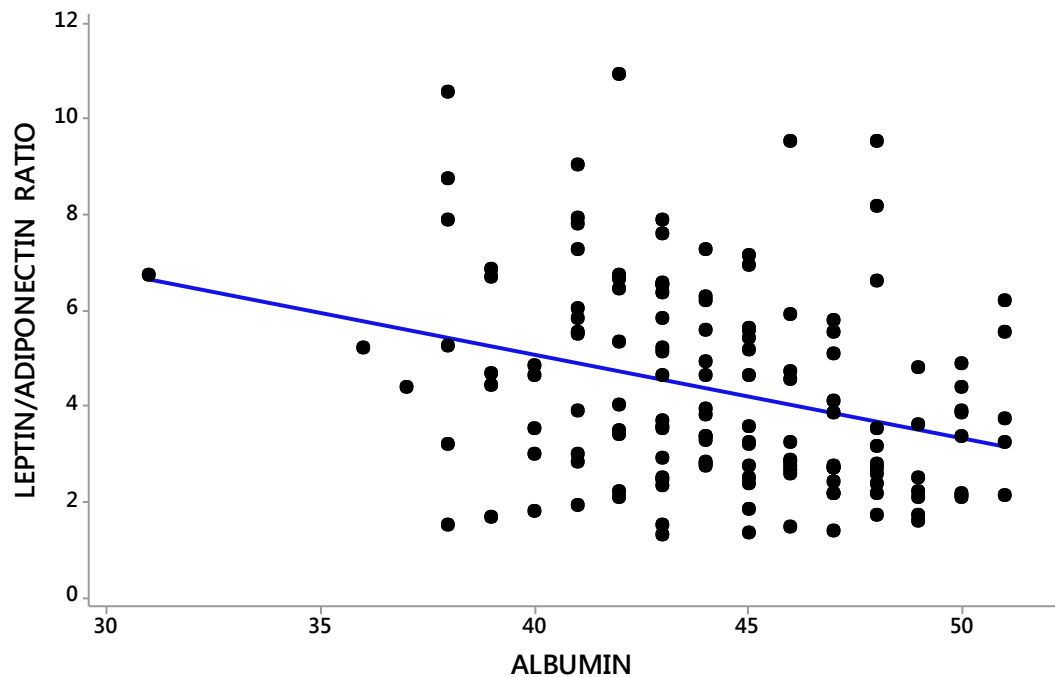
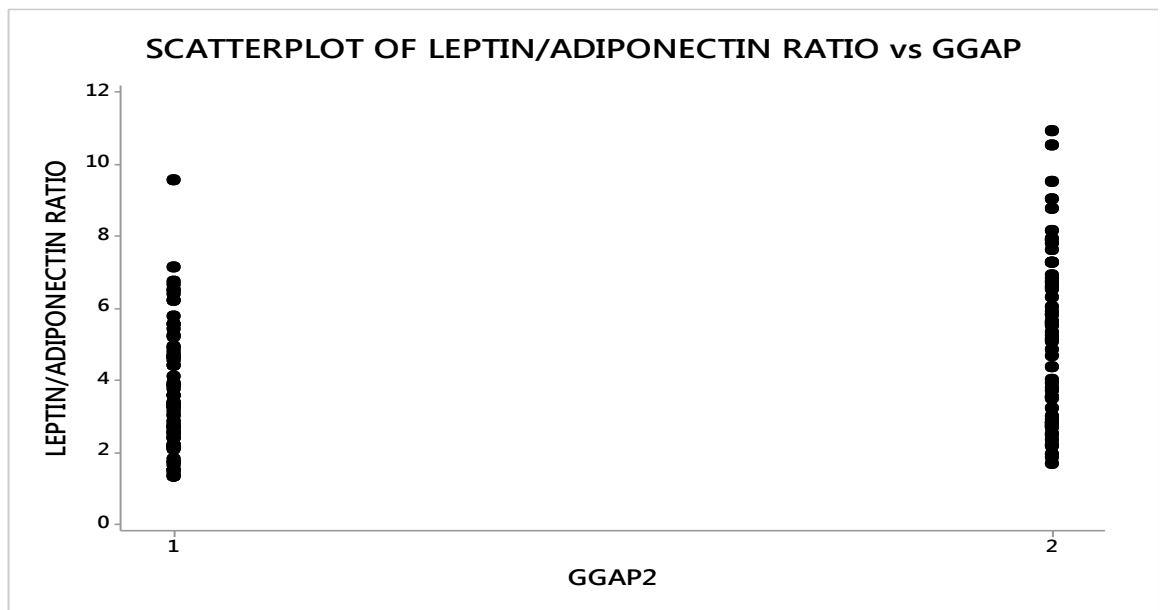


Figure 5.7 Scatterplot of the distribution of Leptin/Adiponectin ratio plotted against albumin levels in the blood of patients. This shows a negative linear relationship

Figure 5.7 shows that there is a weak negative correlation ($r = -0.306$ and $p < 0.001$) between L/A ratio and the albumin levels in the blood of patients showing the link between L/A and renal complications.

5.3.9 Correlation of leptin/adiponectin ratio with GGAP

a)



b)

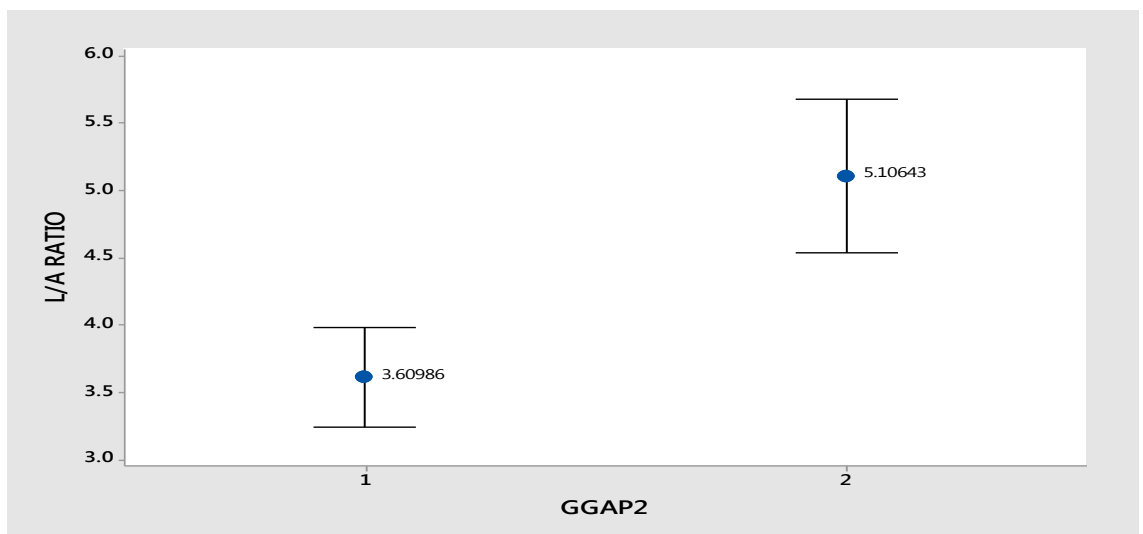


Figure 5.8 Scatterplot and interval plot of the distribution of L/A ratio plotted against G-Gap (positive and negative) a) Scatter plot of L/A ratio plotted against positive and negative G-Gap, b) Interval plot of L/A ratio plotted against positive and negative G-Gap. (1) is negative and has a lower mean (3.6) L/A ratio than (2) which is positive with a higher mean (5.1).

Figure 5.8 shows that there is a weak positive correlation ($r= 0.356$ and $p<0.001$) between L/A ratio and the G-Gap of patients. The scatterplot shows the patients with the negative G-gap(1) having lower L/A ratios than those with Positive GGAP(2) showing that negative G-Gap may be associated with less complications than positive GGAP. Also the interval plot shows a lower mean for the patients with a negative G-gap than those with positive G-Gaps.

5.3.10 Effect correlation of leptin Vs BMI, HDL, GGAP, Adiponectin, Albumin and eGFR

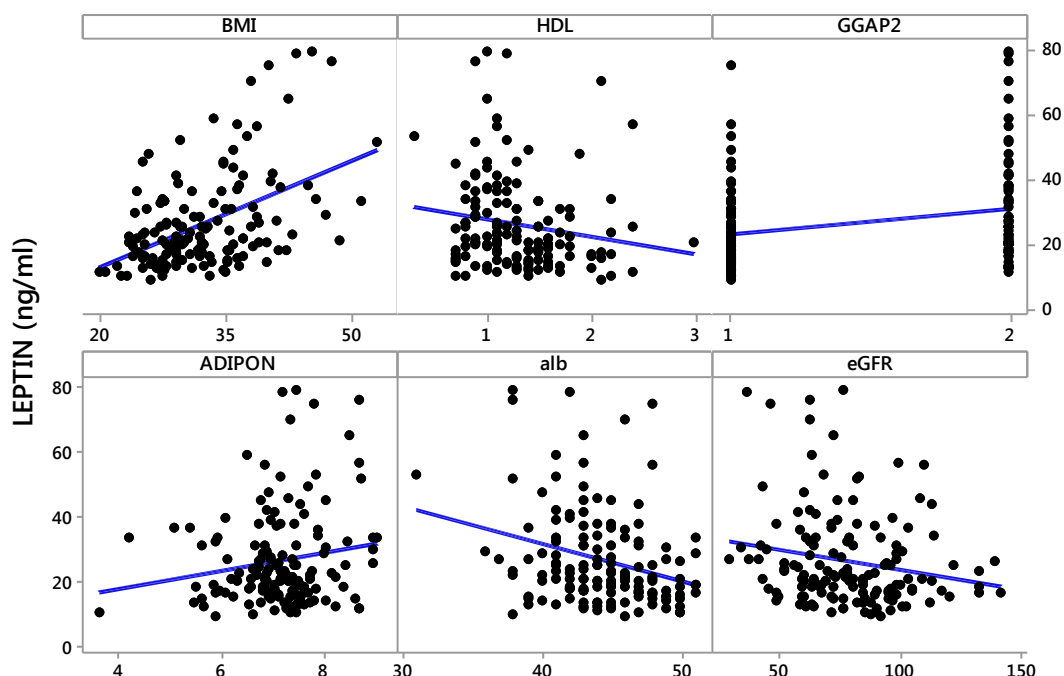


Figure 5.9 Scatterplot of Leptin levels vs. BMI, HDL, GGAP, Adiponectin, Albumin, and eGFR.

Figure 5.9 shows that there is a weak positive correlation between leptin and BMI ($r = 0.473$, $p < 0.001$) a weak negative correlation between leptin and HDL ($r = -0.165$, $p < 0.048$), weak positive correlation between leptin and GGAP ($r = 0.266$, $p < 0.001$), weak correlation with adiponectin ($r = 0.166$, $p < 0.046$), weak negative correlation with albumin (-0.287 , $p < 0.000$), and a weak negative correlation with eGFR -0.189 , $p < 0.023$.

5.3.11 Correlation of Adiponectin VS BMI, HDL, CVD risk, leptin and albumin

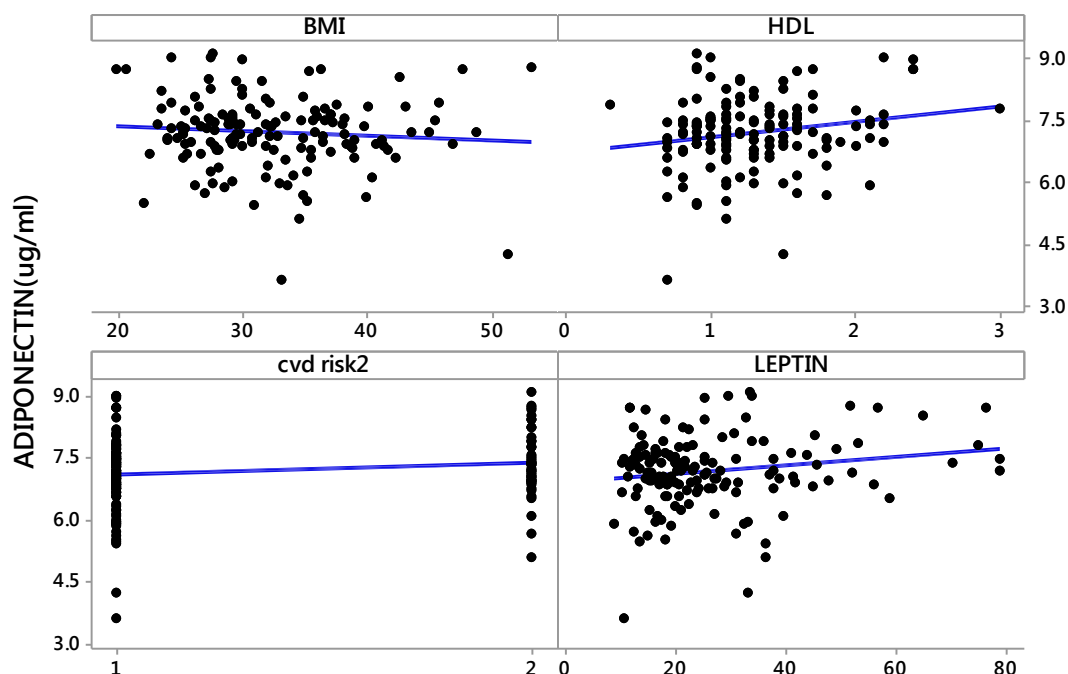


Figure 5.3.10 Scatterplot of Adiponectin levels vs. BMI, HDL, CVD risk, Albumin, and eGFR.

Figure 5.3.10 shows that there is a weak negative correlation between adiponectin and BMI ($r = -0.083$, $p < 0.322$) a weak positive correlation between adiponectin and HDL ($r = 0.188$, $p < 0.024$), weak correlation with leptin ($r = 0.166$, $p < 0.046$), weak positive correlation with CVD risk ($r = 0.161$, $p < 0.048$) with those with primary risk (1) having a wider range (3.8 to 9) than the secondary risk (4.7 to 9), and a weak negative correlation with eGFR9 -0.189 , $p < 0.023$.

5.3.4 Discussion

The preceding chapters have explored the role of leptin and adiponectin in the regulation of beta cell and endothelial cell function. Obesity has been linked to T2DM and CVD via adipokines (Bravo *et al.*, 2006, Barnes 2011). The adipokines of interest in this study are leptin and adiponectin and the leptin/adiponectin ratio has been used as an index for assessing the risk of developing complications in CVD and insulin resistance (Ling *et al.*, 2004). Leptin and adiponectin on their own were both suggested to reduce insulin resistance and due to the fact that leptin increases with obesity and adiponectin reduces, the leptin and adiponectin ratio has been used as a more objective method to assess insulin resistance and predisposition to cardiovascular diseases (Oda *et al.*, 2007).

Another factor which has been associated with an altered risk of diabetic complications is the G-gap (this is a measure of the deviation of glycated HbA_{1c} away from its expected value, such that a negative G-gap is taken as meaning a lesser level of glycation than expected and a positive G-gap meaning a higher level of glycation of proteins) (Nayak *et al.*, 2013).

Previous studies have suggested that the leptin/adiponectin ratio (LAR) is linked to insulin resistance and insulin resistance has been linked to obesity,

hypertension, atherosclerosis and diabetes (Rashid 2013; Assiri and Kamel 2012). Also attempts have been made over the years to find a good parameter for measuring glycaemic control as glycaemic control or appropriate glucose homeostasis is the main key to preventing diabetic complications (Nayak *et al.*, 2013). Glycated haemoglobin (HbA_{1c}) has been used for a long time (over 40 years) as it was considered to reflect glycaemia over the preceding 12 weeks (WHO 2011). Glycated haemoglobin is formed from non-enzymatic concentration-dependent covalent bonding of glucose to haemoglobin within erythrocytes, however, fructosamine measures the plasma concentration of glucose over a period of 2-3 weeks (Rashid 2013). Previous studies have however suggested that due to the fact that many factors influence the protein's turn-over (erythropoietic processes, blood and protein losses), HbA_{1c} may not be as reliable as it has always been thought to be by previous studies (Nayak *et al.*, 2013). Further studies have shown that in contrast to the non enzymatic process of protein glycation, there is also the fructosamine 3-kinase enzyme that deglycate protein within the cell (including most probably HbA_{1c}) (Nayak *et al.*, 2013). Although fructosamine has the potential to be used as a parameter to assess glucose homeostasis, it is not without its issues such as the fact that it is only an indirect measure of glycation of plasma proteins such as albumin, and that it doesn't predict glucose homeostasis for as long a period as HbA_{1c}.

Because of this, the concept of G-gap, even after having been associated with an altered risk of diabetic complications still has a number of issues over its relevance in the assessment of glycaemic control (Sacks *et al.*, 2011). Therefore, in the present study, a new attempt at finding parameters for

assessing the possible risk of complications arising from diabetes was developed by comparing or correlating the G-gap to the leptin/adiponectin ratio.

The clinical aspect of the present study, therefore aimed to look at the following critically; the correlation between the leptin/adiponectin ratio and the parameters linked with complications in diabetes such as BMI, cholesterol levels, HDL levels, eGFR, albumin. Also the individual correlation of leptin and adiponectin with the parameters linked with complications in diabetes. This comparison was made to see if the leptin/adiponectin ratio is a better predictor of complications in diabetes.

Previous studies suggest that a positive G-gap correlates well with higher levels of complications, while a negative G-gap in diabetic patients correlates with lower levels of complications (Nayak *et al.*, 2013). Therefore, in the present study, the G-gap is used as an important predictor of risk of complications. The leptin/adiponectin ratio has been shown to be predictor of atherosclerosis, insulin resistance and diabetes risk and is said to be a better predictor of T2DM complications and CVD (Kappelle *et al.*, 2012).

The result from the present study shown in figure 5.3 demonstrates that diabetic patients with negative G-gap had lower LAR (mean 3.204) than those with positive G-gap (mean 4.611). This supports the hypothesis that the higher the

L/A ratio, the higher the risk of developing complications as a positive G-gap has been linked with greater risk of complications as suggested by Nayak *et al.* (2013).

The studies by Ling *et al.* (2014) and Oda *et al.* (2007), highlight the link between LAR and BMI. In the present study, the results in figure 5.4 shows a significantly strong positive linear correlation between leptin/adiponectin ratio and the body mass index of the patients ($r = 0.752$ with $p < 0.0001$). This shows support for the hypothesis that individuals with high leptin/adiponectin ratios will be likely to have higher levels of insulin resistance as the higher the LAR, the higher the BMI (and the higher the BMI the greater the insulin resistance.) (Ling *et al.* 2014). These results also highlights the suggestion by Oda *et al.* (2007) and Rashid (2013) that LAR is better at predicting risk factors for complications as the correlation between LAR and BMI is higher than the correlation between leptin and BMI or adiponectin and the BMI (seen in figures 5.9 and 5.10). These figures also show that just like the LAR, leptin has a direct linear relationship with the BMI, while adiponectin has a negative linear relationship with BMI though not significant which is in agreement with the studies by Bahathiq (2010) and Oda *et al.* (2007). These results are also consistent with the findings in chapter 3 and 4 and support the hypothesis which suggest that the higher the BMI, the higher the leptin levels (a positive linear relationship) and *vice-versa* for low BMI. Also it suggests that the higher the BMI the lower the adiponectin and *vice-versa* for low BMI (a negative linear relationship) (Oda *et al.* 2007). The relevance of this finding is the possibility of using LAR as another

parameter for predicting the risk of developing complications as BMI has been used over the years.

In the present study therefore, the complications considered are the commonest ones associated with diabetes which are CVD complications, renal and retinal complications.

In the assessment of the CVD complications, tables 5.1 and 5.2 show that LAR and leptin correlated positively with CVD risk (weakly and non-significantly) while adiponectin is negatively correlated with CVD risk (weakly but significantly $r = -0.161$, $p < 0.048$). This is in agreement with studies that suggest that high adiponectin reduces the risk for CVD (Zhu *et al.*, 2008) as the present study shows that the higher the levels of adiponectin, the lower the risk of CVD. This is in contrast to the views of Vega *et al.* (2013) and Vasilescu *et al.* (2010) and Kapelle *et al.* (2012) which suggest that LAR is a better predictor of CVD risk than adiponectin since the present study shows that adiponectin better correlates negatively with a reduction in CVD risk. One of the possible reasons for the differences seen in the results is the fact that Kapelle *et al.* (2012) used only men in their studies unlike the current study. Also, in the present study, the classification of the risk of CVD was based on the presence or absence of any previous cardiac, cerebral or peripheral macrovascular event unlike the study by Vasilescu *et al.* (2010) in which the CVD risk was based on CIMT (carotid intima-media thickness).

In the present study, the parameters for monitoring or assessing the risk for renal diseases (eGFR, albumin), CVD (Cholesterol, HDL) and liver function (albumin) were also considered and correlated with the LAR, adiponectin and leptin levels in the patients plasma. Table 5.1, 5.2 and 5.3 show the various r (correlation coefficient) and p-values of the correlation of LAR, adiponectin and leptin levels with the various parameters.

With respect to renal function, leptin levels were significantly negatively correlated (weakly, $r=-0.189$ and -0.287 respectively) to the eGFR and the albumin levels (with no correlation between LAR and eGFR but only with albumin) (Table 5.1 and 5.2). This suggests that the LAR may not be as strong in predicting renal dysfunction in diabetic patients as leptin. This suggests that the higher the levels of leptin and LAR the lower the eGFR suggesting that diabetics with high levels of leptin and LAR may be more predisposed to developing renal complications which is in agreement with Chou *et al.* (2013). The result in table 5.3 shows no significant correlation between adiponectin and the parameters that represent the renal function (negative correlation to eGFR and positive correlation to albumin). This is in disagreement with the study of Jung *et al.* (2012) where it was reported that adiponectin has an inverse relationship with nephropathy. It is however important to note that there have been a lot of controversies concerning the outcomes of results from previous studies and the reasons that have been put forward by Jung *et al.* (2012) may apply to the conditions chosen for the present study. The reasons suggested are the fact that the differences may be due to differences in study populations

according to the various diagnostic criteria. Also, it was suggested that different distributions of the severity of the microangiopathies and ethnic variations in the selected volunteers may be reasons for the differences in results. In the present study, the ethnic origins have not been taken into consideration.

With regards to retinopathy, the LAR, leptin and adiponectin did not significantly correlate with the retinopathy levels seen in patients and therefore the results suggest that LAR is not a very good predictor of retinopathy risk in diabetic patients in contrast to the study by Kato *et al.* (2008) which suggested that the levels of adiponectin were higher in those with retinopathy than those diabetics without retinopathy and that of Jung *et al.* (2012) that found that adiponectin was lower in those with nephropathy. The LAR however has a linear positive correlation with retinopathy as against the leptin and adiponectin levels which supports the hypothesis that LAR may be a better predictor of retinopathy than either of leptin or adiponectin.

This study has provided support for a number of theories. The results from this study confirm that the adipose tissue is definitely more than an energy storing organ as the adipokines released from them (in this case leptin and adiponectin) correlates with risk of developing CVD or renal complications in diabetic patients which agrees with the research by Koves *et al.* (2008). Also, results from this study show that leptin and the LAR are good predictors of the implications of high BMI on the CVD and renal complications in diabetics as

suggested by Vega *et al.* (2013) and Ling *et al.* (2014). Also the result in this study support the theory that high adiponectin may prevent CVD.

It is however surprising that adiponectin appears to have very little implication in the possible risks of the development of retinopathy and nephropathy in contrast to the findings of Jung *et al.* (2012) which suggested that the patients with nephropathy had low adiponectin while those with retinopathy had high concentrations of adiponectin.

This study therefore shows that clinically, if interventions can be made to increase the levels of adiponectin and reduce the levels of leptin, there is a high possibility of reducing the risk of development of CVD and renal complications in diabetic patients.

In conclusion, this chapter has considered the relevance of the LAR in the development of complications in diabetic patients by correlating the assayed levels of leptin and adiponectin ratio with the predictors of complications in diabetic patients. This study shows that the LAR linearly correlated with the BMI which suggest that the LAR is a good indicator of all the issues that may be linked with having high BMI. Also this study shows that leptin is better at predicting renal complications and adiponectin better at predicting CVD complications in diabetic patients than the leptin/adiponectin ratio.

Chapter 6

Final discussion, conclusion and implications for future research studies

6.1 Final Discussion

Obesity has been shown to now affect about 520 million people and has been linked with type 2 diabetes mellitus (WHO 2014 review). It is estimated that there are about 382 million people (between the ages 20 and 79) worldwide affected by diabetes and in the UK, about 3.2 million people are affected (DUK 2014). Despite the fact that it has been suggested that obesity is linked to T2DM and CVD, the basis of this link is not completely understood. Due to the increasing incidence of obesity and the corresponding increase in T2DM and CVD, it is important to note that it has been demonstrated that adipose tissue, which used to be seen only as a metabolic storage tissue, also has endocrine functions and produces proteins termed adipokines that influence metabolism in the human body (Trayhurn and Wood 2004).

In the present study, the opposing effects of adipokines leptin and adiponectin reported by previous research were investigated to clarify their role as a link between obesity and CVD and T2DM since the basis of their possible role is not well understood. Many of the previous studies have looked at the effect of these adipokines individually but not in combinations and therefore one of the aims of this present study was to explore the effect of these adipokines in combination especially in CVD while for the first time the effect of a combination of adipokines on pseudoislets (clumps of beta cells that are similar to islets of Langerhans and prepared *in vitro*) was reported in this study. Also one of the

aims of this study was to explore the impact of the leptin/adiponectin ratio on the complications seen in diabetic patients and to examine the role of adipokines in amylin secretion.

In the present study, the results illustrated in chapter 3 show the potential role of leptin and adiponectin in the pathogenesis of CVD. Angiogenesis and atherosclerosis have been shown to be important processes in the pathogenesis of CVD (Lip 2004), and therefore the effect of leptin and adiponectin on genes that code for angiogenesis (MMP2, TIMP2, TIMP3) and atherosclerosis (LPA, LPL) was explored.

Prior to this study, there have been attempts to explore the effects of leptin and adiponectin on the angiogenic and atherosclerotic genes in order to better understand the pathogenesis of CVD, but very little was understood of the effect of combining these adipokines (leptin and adiponectin) on atherosclerotic and angiogenic genes. Also the basis of the effect of the adipokines in the process of pathogenesis of CVD is not completely understood.

The result from the present study shows that leptin at high concentrations (at 16ng/ml) increases the expression of all angiogenic and atherosclerotic genes, and has very little effect in lower concentrations (8ng/ml). This suggests that leptin in high concentrations (that is in the region of 16ng/ml which is similar to

that seen in obese individuals) may predispose to CVD while lower concentrations such as those seen in lean individuals poses less risk. This is in agreement with the findings of Dubey and Hesong (2006) and provides evidence to support the suggestion that leptin is one of the links between obesity and CVD. This is important in the management and reduction of obesity-related cardiovascular diseases as a strategy of weight loss which may lead to a reduction in leptin levels could improve the outcomes of CVD risk in obese individuals.

The present study also showed that the treatments with high adiponectin (3.6µg/ml in the range observed in lean individuals) caused an increase in the expression of Tissue Inhibitors of Metalloproteinases 2 and 3 (TIMP2 and 3) and an inhibition in the expression of the Matrix Metalloproteinase 2 and an inhibition of the atherosclerotic genes. At lower concentrations of adiponectin, the results were not as significant and consistent as those with a high adiponectin concentration but followed a similar pattern to that observed in the latter. A number of studies have suggested that MMP2 plays a key role in the pathogenesis of CVD while TIMPs modulate this effect. The results from the present research therefore suggest that adiponectin at high concentrations (as seen in lean individuals) may lead to a reduction in the risk for angiogenesis and atherosclerosis linked with CVD.

The data from the current work also shown that adiponectin in high concentrations (3.6µg/ml) when combined with leptin (16ng/ml) is able to reduce the up-regulatory effect of leptin on atherosclerotic and angiogenic genes, but lower concentrations of adiponectin (36 ng/ml) did not have the same effect on the up-regulatory effect of leptin on angiogenic and atherosclerotic genes. This is in agreement with the study by Handy *et al.* (2011) but this study provides further evidence of the effects of low leptin and low adiponectin in combinations which is lacking in previous studies. It is interesting also to see, however, that the real time PCR results suggests that combining high leptin with low adiponectin seems to have an additive effect in the expression of many of the angiogenic and atherosclerotic genes, (except MMP2 showing consistency in the effect of adiponectin on its own and in combinations) a result which may suggest that in obese individuals, the individual actions of leptin on angiogenic and atherosclerotic genes may be accentuated leading to even more risk for CVD.

The observations from the present work support the model which suggests that high levels of adiponectin as seen in lean individuals may have a protective effect on the cardiovascular system. The results also suggest that individuals with low adiponectin as seen in obesity may be more predisposed to CVD. This is important for further research into possible adiponectin therapy that can reduce the effect of leptin on the risk for developing CVD, especially in obese people with high levels of leptin.

Also in the current study the role of AMPK, ERK and P38 MAPK in the mechanism of leptin's and adiponectin's effect was explored. The three enzymes were shown to be involved in the effects of leptin and adiponectin on angiogenic genes which is in agreement with the work of Lee *et al.* (2008) which suggests the role of ERK in the effect of adiponectin and Chandraseker *et al.* (2008) which suggested the role of AMPK. It is however surprising that the role of AMPK in the effect of adiponectin was not as clear as has been reported by some previous studies (Kim *et al.*, 2009, Zhang *et al.*, 2012, Mattu and Randeva 2012).

Many researchers including Ley *et al.* (2009), Mattu and Randeva (2013), Bravo *et al.* (2006) and Barnes (2011) have suggested that leptin and adiponectin as adipokines are an important link between obesity and CVD and diabetes mellitus. The data in chapter 3 showing the effects of leptin and adiponectin on angiogenic and atherosclerotic genes (thus CVD), agrees with this suggestion and it was logical to explore the effect of leptin and adiponectin in the beta cell functions in order to better understand the role of obesity in diabetes mellitus.

Insulin and amylin secretion were used as parameters to assess beta cell function as the most important functions of the beta cells relate to the secretion of insulin and amylin; the latter is said to be co-secreted with insulin and to affect the function of the hormone in many tissues as it is related to insulin resistance (Karlsson *et al.* 1998). Therefore it was important to be able to

effectively measure the secretions of insulin and amylin from treated beta cells *in-vitro*. Most researchers in the past have used beta cells grown in monolayer cells, but the study by Persaud *et al.* in 2010 suggested that growing the beta cells into “pseudo-islets” allows the cells to mimic real islets of Langerhans. Beta cells are reported said to produce insulin in a more physiological way and also in larger quantities when they work together (as in the islets of Langerhans or the pseudo-islets), than when they produce insulin as monolayer beta cells possibly as a result of different cell to cell interactions. Therefore, in this study, pseudo-islets were used to explore the effects of leptin and adiponectin on beta cells.

Results from the current study agrees with the suggestions by Persaud *et al.* (2010) as the insulin secretion by the pseudo-islets was about an average of 2 folds the secretion seen in monolayer beta cells and there appeared to be a better step-wise glucose response and better control secretion in the pseudo-islet.

The data show that leptin at 200 to 500 ng/ml appear to have a significant inhibitory effect on insulin secretion with an inverted “U” shape in contrast to the reports described by Dunmore and Brown (2013). This is also consistent with the normal function of leptin *in-vivo* in which leptin at reported concentrations (5-15ng/ml) increases insulin secretion and in which very high leptin concentrations are said to inhibit insulin secretion (Zhao *et al.* 1998). This

result is consistent for the cells treated in low and high glucose thus suggesting that the two extremes of the diabetic glucose derangements may have similar implications on the resulting factors which lead to diabetes and which complicate diabetes. The importance of this is similar to that relating to the role of leptin in CVD as discussed in chapter 3, as this study suggests that a reduction in weight in an obese individual may lead to a reduction in the leptin levels and therefore a boost for insulin secretion and a reduction in diabetic complications and even a delay in the onset of diabetes.

This study also shows that 1 and 5µg/ml of adiponectin in both low and high glucose increased the secretion of insulin significantly. This is in agreement with the study by Okamoto *et al.* (2008) and Seufert *et al.* (2004). This result suggest that even at low concentrations (as seen in obesity), adiponectin may still increase insulin secretion but not significantly in the present study, suggesting that any measures that can boost adiponectin levels may boost insulin secretions. This therefore underlines the possible benefit of weight loss in the management of CVD and diabetes as it has been shown that adiponectin levels are higher in lean individuals than in obese individuals.

The result of the current study may also support a role for amylin on insulin secretion. The normal molar insulin: amylin ratio is about 100:1 but for the beta cell treatments, the average ratio was between 8:1 to 25:1 which suggests that diabetic models reflected in the design of the experiments resulted in high

amounts of amylin secretion which may inhibit insulin secretion. The result shows that adiponectin in low glucose, appears to increase the secretion of both insulin and amylin and that there appears to be no corresponding effect of high amylin levels on insulin levels as suggested by Tedstone *et al* (1990). This is however the opposite of what was found in high glucose and in the leptin treatment in low and high glucose. In the present study, there was an opposite corresponding secretion of amylin and insulin which may suggest that the amylin may be contributing to the effect on insulin secretion. This may suggest why there is more insulin secretion by adiponectin in high glucose and why leptin is consistently inhibited in low and high glucose as it may be as a result of contributions by amylin. The relevance of this to clinical practice may be seen in the role of adiponectin in possibly altering the insulin: amylin ratio in a beneficial way (more insulin less amylin). This could suggest potential pharmaceutical approaches to treating diabetes by boosting insulin and reducing amylin secretion (thus also reducing the collection of amylin in tissues and the formation of amyloid which may damage beta cells and reduce insulin secretion as a result of fewer numbers of beta cells).

As leptin had the most consistent effect, the basis of these effect were investigated by the exploration of PP-1 gene expression which is suggested to be the gene that leptin blocks in order to prevent the influx of calcium into beta cells, thus inhibiting insulin secretion. The result from the present study show that treatment with 500ng/ml of leptin in low glucose caused a significant inhibition in PP1 gene expression (as mRNA) which may be the basis for the

effect of leptin on insulin secretion at 500ng/ml. In high glucose, however the opposite was found as 500ng/ml of leptin caused a significant increase in insulin secretion,

Most of the studies done initially were in-vitro. As suggested by Mattu and Randeva (2012), *in vitro* studies may not reflect all the interplay between the adipokines and the human body in obesity, CVD and diabetes. Therefore in the current study, diabetic patients were recruited and blood samples collected from them in order to measure the levels of leptin and adiponectin in the blood so as to be able to correlate this with the parameters that suggest risk linked to developing complications in diabetes (those such as renal, retinal and CVD complications).

As shown in chapter 5 in the present study, leptin:adiponectin ratio (LAR) was examined as a parameter to predict the risk for complications since other studies suggest that because the amount of adiponectin and leptin varies with obesity (and amount of adipocytes.) the LAR was a more objective measure (Oda *et al.*, 2007).

The results showed that both the LAR and leptin were positively linearly related with the BMI with strong correlation factors of 0.752 (p-value <0.0001) and 0.779 (p-value<0.0001) respectively. This may suggest that people with higher

BMI are likely to have a higher LAR and therefore be more predisposed to the complications related to obesity and high levels of leptin. When considering the complications, retinal, CVD and renal complications which are the commonest seen in diabetic patients were examined for correlation with the measured adipokine levels. Adiponectin was mostly negatively correlated with most of the risk parameters, but had a significant weak correlation with CVD ($r = -0.161$ p -value < 0.05) which does not entirely agree with the view put forward by Vega *et al.* (2013) which states that LAR is a good predictor of CVD risk. Adiponectin also has little implication in the present study in relation to the risks of developing retinopathy and nephropathy as compared with the suggestions by Jung *et al.* (2012).

This part of the study re-iterates the fact that the adipose tissue is more than just a storage organ as it is an active tissue with wide-ranging metabolic effects on other tissues through the secretion of adipokines. It is important to also note that the LAR of patients with negative G-Gaps (associated with lower incidence of complications) was lower (mean 3.204) than the LAR of those with positive G-Gap (associated with more complications) with a mean of 4.611.

This therefore suggests that the LAR, according to the result is not as good in predicting the retinal and renal complications complication but is relevant in predicting the CVD risk as a result of its correlation with BMI and HDL and high leptin.

6.2 Conclusion

The present study was carried out to explore the role of obesity in the development of diabetes mellitus and cardiovascular disease as it was considered that the increasing incidence of obesity may be related to that of CVD and T2DM. This was done by assessing the concentrations of leptin and adiponectin (individually and in combination) that has effects on beta cell and endothelial cell function in order to ascertain the most effective exact concentrations of leptin and adiponectin that affect beta cell and endothelial cell function. Another aim of this study was to explore the role of amylin in type 2 diabetes and also the effect of combining the adipokines.

Previous literatures have reported on some of these issues and have not been conclusive on some issues which form the questions for the current study. One of our questions explored what concentrations of leptin and adiponectin have an effect on beta cells and endothelial cells and also what the effect is. Also the current study looked into what the effect of the combining leptin and adiponectin will be and another area of interest was to ask whether there is a correlation between the concentrations of leptin and adiponectin in the diabetic patient and the complications seen in patients and also to ask if there is a correlation between the effect of leptin and adiponectin in the patients and the effects seen in the lab.

This study has shown that leptin in high concentration (16ng/ml), predisposes to CVD by increasing the expression of angiogenic genes and atherosclerotic genes, while adiponectin in high concentrations(3.6µg/ml) reduces the risk of cardiovascular disease by reducing the expression of some of the atherosclerotic and angiogenic genes and promoting the expression of the anti-angiogenic genes. Also for the first time it has been shown consistently that there is a significant reduction in the expression of the angiogenic and atherosclerotic genes when high levels of adiponectin and low levels of leptin are combined. Also, this study has been able to show that AMPK, ERK and P38 enzymes are involved in the function of leptin and adiponectin.

For assessment of T2DM, this study has shown that leptin inhibits insulin secretion while adiponectin increases it. Also it was shown that the insulin /amylin ratio of the treatments were quite high and that leptin increases amylin secretion. Adiponectin (5µg/ml) is shown to increase insulin secretion and reduce amylin secretion. This may be relevant in the development of therapeutic measures that may increase adiponectin secretion, thereby altering the insulin/amylin ratio to favour a boost in insulin secretion in diabetics.

Also it has been shown in this study for the first time that high leptin/adiponectin ratio correlates with indicators that suggest risks of complications in diabetes patients.

Overall the evidence provided suggests that leptin increases the risk for CVD and T2DM while adiponectin reduces the risk of CVD and promotes insulin secretion and reduces amylin secretion, thereby possibly improving or preventing T2DM. More interestingly, a high concentration of adiponectin is shown to reduce the risk of CVD. This will make an interesting basis for the development of pharmacologic interventions for T2DM.

It is clear from this study that leptin potentially predisposes to CVD and T2DM while adiponectin on its own and when combined with Leptin reduces the risk of CVD and T2DM.

6.3 Future Research

The results from this study show that leptin promotes the development of CVD and T2DM and adiponectin discourages it and the basis for this has been explored. The results also shows that a combination of the adipokines appear to inhibit the risk of CVD.

With regards to CVD, more research needs to be done to explore the basis for the reduction in the expression of angiogenic and atherosclerotic genes when leptin and adiponectin are combined. Also further research needs to be done on the role of varying concentrations of adiponectin on the expression of angiogenic and atherosclerotic genes as low concentrations of adiponectin appears not to have consistent effects unlike high concentrations of adiponectin.

With regards to diabetes mellitus, more studies need to be done to find out the effects of combining adipokines on insulin and amylin secretion. Also more research needs to be done on the effect of the absence of glucose on beta cells as the results from this study suggest that the absence of glucose destroys the beta cells leading to leakage of insulin and high levels of insulin in samples with no glucose. Also some very high concentrations of glucose (22mM) appeared not produce the expected response in insulin secretion when compared to

some lower concentrations (15 mM) and higher concentrations (30mM). More studies may need to be done to find out why these concentrations of glucose were not sensed by beta cells as this may be important for selecting glucose concentrations to treat cells with in future studies. Also, the effect of amylin on insulin secretion is not very clear from this study and for the purposes of future research, more research needs to be done to explore the direct effect of amylin on insulin secretion as the results from this study show that the cells have very high levels of insulin/amylin ratio.

Chapter 7

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Appendices

Appendix1

Appendix 1.1 Equipment, specialized kits and reagents and chemicals for beta cells experiments

1.1.1 Equipments for beta cell experiments

Centrifuges	M.S.E Mistral 200,Fision, Instruments U.K.
CO2 Incubator	Jensons Scientific Inc, Bridgeville, PA 15017, USA.
Inverted Microscope Celti Belgium.	Optical Instruments,
Magnetic Stirrer Hotplate	Stuart Scientific, Beacon Road Staffordshire, UK
Platform Shaker STR6	Stuart Scientific, Beacon Road, Staffordshire, UK.
Vortex	Sigma-Aldrich Company Ltd, Dorset, UK.

Waterbaths
(Cambridge)Ltd,

Grants Instruments

Cambridge, UK.

1.1.2 Specialized kits for beta cell experiments

Mouse insulin secretion ELISA kit

Mercodia, Upsalla Sweden.

1.1.3 Reagents and chemicals for beta cell experiments

Beta Mercaptoethanol

Bio-Rad Laboratories Ltd,
Hemel Hempstead, UK.

DMSO

Sigma-Aldrich Company Ltd,
Dorset, UK.

Hanks Buffered Salt Solution

Sigma-Aldrich Company Ltd,
Dorset, UK.

L-glutamine

Sigma-Aldrich Company Ltd,
Dorset, UK

Penicillin-Streptomycin Solution

Sigma-Aldrich Company Ltd

Dorset, UK

RPMI 1640 without Glucose

Sigma-Aldrich Company Ltd,

Dorset, UK

Trypsin/EDTA

Sigma-Aldrich Company Ltd,

Dorset, UK

Appendix 1.2 Equipments. Specialized kit and reagent and chemicals for endothelial cell experiments

1.2.1 Equipments for endothelial cell experiments

Block Heater

Wolf laboratory,

York,UK

Centrifuges

M.S.E Mistral 200,

Fision Instruments, U.K.

CO2 Incubator

Jensons Scientific Inc.

Bridgeville, PA 15017,USA

Coulter Counter Z1

Beckman Coulter Limited, High

Wycombe, UK

Hybaid PCR Express Thermocycler

Hybaid, Action Court,

Middlesex, UK

Inverted Microscope Instruments,	Celti Optical Belgium.
Magnetic Stirrer Hotplate	Stuart Scientific, Beacon Road, Staffordshire, UK.
Platform Shaker STR6	Stuart Scientific, Beacon Road, Staffordshire, UK.
Vortex	Sigma -Aldrich Company Ltd. Dorset, UK
Waterbaths (Cambridge)Ltd.	Grants Instruments Cambridge, UK

1.2.2 Specialized kit for endothelial cell experiment

Precision Nanoscript Reverse Transcription Kit.	Primer design Northampton, UK
Total RNA Purification Kit. Corporation	Norgen Biotek Thoroid, Canada

1.2.3 Chemicals and reagents for endothelial cell experiments

Bovine serum albumin	Sigma-Aldrich Company Ltd. Dorset, UK
dNTP mix	Promega UK, Southampton, UK
Ethidium bromide	Sigma-Aldrich Company Ltd, Dorset, UK.
Fetal Bovine Serum	PAA Laboratories Ltd, Yeovil, UK
Hanks Buffered Salt Solution	Sigma-Aldrich Company Ltd, Dorset, UK
Hyper Ladder- 100bp	Bioline Ltd, London, UK
L-glutamine	Sigma-Aldrich Company Ltd, Dorset, UK
Low EEO agarose gels('Hi-Pure')	BioGene Ltd, Kimbolton, UK
Nuclease-Free Water	Promega UK,

Southampton, UK

Penicillin-Streptomycin Solution	Sigma-Aldrich Company Ltd, Dorset, UK.
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RPMI 1640 without Glucose	Sigma-Aldrich Company Ltd, Dorset, UK.
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Trypsin/EDTA	Sigma-Aldrich Company Ltd, Dorset, UK
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1.3 Equipment, specialized kits, chemical and reagents for clinical research

1.3.1 Equipment for clinical research

Roche Modular P analyzer	Roche Diagnostics Ltd., West Sussex, UK
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Tosoh G7 analyzer (HbA _{1c})	Tosoh Bioscience Ltd, Worcestershire, UK
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1.3.2 Specialized Kits for clinical research

Duaset Elisa kit for Human Adiponectin.	R&D systems Europe Ltd Abingdon, UK
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Duoset Elisa kit for Human Leptin.

R&D systems Europe Ltd

Abingdon, UK

1.3.3 Reagents and Chemicals for clinical research

PBS - 137 mM NaCl, 2.7 mM KCl, 8.1 mM Na₂HPO₄,

1.5 mM KH₂PO₄, pH 7.2-7.4, 0.2 mm filtered.

Wash Buffer - 0.05% Tween[®] 20 in PBS, pH 7.2-7.4

Reagent Diluent¹ - 1% BSA in PBS, pH 7.2-7.4, 0.2 mm filtered. Quality of BSA is critical (see Technical Hints).

Substrate Solution - 1:1 mixture of Color Reagent A (H₂O₂) and Color Reagent B (Tetramethylbenzidine).

Stop Solution - 2 N H₂SO₄

Appendix 2

Table 3 Serial dilution of standard stock solution

Standard No.	Std. volume	1x Assay Buffer	Concentrations
Stock	1000ul	-----	1000ng/ml
#1	100ul of stock	900ul	100ng/ml
#2	100ul of #1	900ul	10ng/ml
#3	100ul of #2	900ul	1ng/ml
#4	100ul of #3	900ul	0.1ng/ml
#5	100ul of #4	900ul	0.01ng/ml

Appendix 3

Conference Presentations

Conference presentations

Majebi, A.O, Dunmore, S.J., Brown, J.E.P. (2014) Role of adipokines leptin and adiponectin in obesity and diabetes mellitus related cardiovascular disease. *Newzealand Society for the Study of Diabetes (NZSSD) Annual Scientific Meeting, Queenstown, Newzealand 2014.*